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THE UNIVERSITY OF ALBERTA

GONADOTROPIN-RELEASING HORMONE SERUM BINDING PROTEIN AND METABOLISM IN GOLDFISH

by

YUAN-PING HUANG

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

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ABSTRACT

In this study, the metabolism of gonadotropin-releasing hormone (GnRH) and the involvement of a specific GnRH binding protein in goldfish serum were investigated. The binding of salmon GnRH (sGnRH) and its superactive analog, [D-Arg^6, Pro^9-NEt]-sGnRH to a macromolecular component of goldfish serum was characterized, using ^{125}I-sGnRH and ^{125}I-[D-Arg6-Pro9-NEt]-sGnRH as labeled ligand. Bound was separated from free labeled ligand by gel filtration with Sephadex G-50 mini-columns. The binding of labeled ligand to goldfish serum was dose-dependent, reversible and specific. The binding was associated with a serum protein, which was isolated from goldfish serum using a nondenaturing polyacrylamide gel electrophoresis (PAGE) method. This binding protein was concentrated in a single band, separated from all major components of serum proteins. The molecular weight of this GnRH binding protein was approximately 40,000 dalton, measured by SDS-PAGE. The isolated GnRH binding protein was found to specifically bind with sGnRH and chicken-II GnRH, the circulating forms of GnRH in goldfish serum identified using the combination of high pressure liquid chromatography and specific radioimmunoassays, but not with mammalian GnRH (mGnRH), chicken-I GnRH or lamprey GnRH. The GnRH binding protein was detected in both sexes of goldfish at different stages of the reproductive cycle, and its titer in serum was not affected by long term treatment of [D-Arg^6, Pro^9-NEt]-sGnRH or E_2 implantation. The binding of GnRH to the binding protein in goldfish serum may affect the clearance of GnRH from circulation. There was good correlation between the half-lives of mGnRH, sGnRH, [D-Arg6, Pro9-NEt]-sGnRH and their affinity towards the binding protein, with the superactive analog [D-Arg6, Pro9-NEt]-sGnRH showing the highest affinity and longest half-life.

ACKNOWLEDGEMENT

I would like to thank Dr. R. E. Peter for his excellent supervision and encouragement throughout this entire postgraduate program. I also wish to acknowledge the members of my supervisory and examination committees, Drs S. Harvey, W. Mackay, N. Sherwood, L. Wang, S. Zalic for their many contributions to my research project.

I am grateful to all the members of the laboratory for their important contribution to the development of my research project, and the subsequent completion of my thesis. The ideas and thoughts generated by the friendly discussions in Journal Club were invaluable. Thanks to every one in the lab, especially Norm Stacy, Carol Nahorniak, Glen Van Der Kraak, J. Chang, Hamid Habibi, Tracy Marchant, Kunimasa Suzuki, Keili Yu, Vance Trudeau, Joseph Dulka, Anderson Wong and Chun Peng. A very special thank to Pat, Terry, Heidi and Bruce for assistance in taking care of the fish. Thanks to my family, especially my dear wife Haiping, for their support.

This research was financed by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to Dr. R. E. Peter. My financial assistance was provided through a scholarship from the TangKakji Trust of Hong Kong, and a Dissertation Fellowship from the University of Alberta.

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1. GENERAL INTRODUCTION

Gonadotropin (GtH) secretion from the pituitary of many teleost fishes is under the dual regulation of gonadotropin-releasing hormone (GnRH) and dopamine, which inhibits GnRH-stimulated release (for review see Peter *et al.*, 1986). Teleosts, in contrast to most other vertebrates, lack a functional hypothalamo-hypophyseal blood portal system. In goldfish, gonadotrophs in the pars distalis (PD) are directly innervated by neurons containing GnRH (Kah *et al.*, 1986) and by neurons containing dopamine (Kah *et al.*, 1984b). In goldfish, the distribution of GnRH cell bodies and neural pathways in the brain have been investigated using immunocytochemical techniques; GnRH cell bodies in the lateral preoptic region are associated with GnRH neural pathways that run through the ventral hypothalamus and the pituitary stalk to terminate in the proximal PD (Kah *et al.*, 1984a; Kah *et al.*, 1984b). In the goldfish the presence of [Trp^7, Leu^8]-GnRH (salmon GnRH, sGnRH) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II, cGnRH-II) in the brain has been demonstrated (Yu *et al.*, 1988); a differential distribution of the two peptides was found, with about equal amounts in forebrain regions, and higher concentrations of cGnRH-II in mid- and hindbrain regions. The presence of a substantial amount of both sGnRH and cGnRH-II in the pituitary of goldfish (Yu *et al.*, 1988) suggests a role for both GnRH forms in the regulation of pituitary functions, including the regulation of GtH release.

GnRH acts directly on the gonadotrophs to stimulate GtH release. Two classes of GnRH binding sites in the goldfish pituitary have been demonstrated, a class of high afffinity/low capacity sites and a class of low affinity/high capacity sites (Habibi *et al.*, 1987). Structure-activity studies for analogs of sGnRH have shown a significant correlation between GtH-releasing potency in vitro and binding to the high affinity sites, but not to the low affinity sites, in the goldfish pituitary (Habibi *et al.*, 1989), indicating

that the high affinity binding sites are involved in the regulation of GtH release.

The activity of several analogs of sGnRH, mammalian GnRH (mGnRH), and [Gln^8]-GnRH (chicken GnRH-I, cGnRH-I) have been studied in goldfish, the sGnRH analog [D-Arg^6, Pro^9-NEt]-sGnRH (sGnRH-A) has been shown to be the most active analog of sGnRH in stimulating GtH release both *in vivo* (Peter *et al.*, 1985), and *in vitro* (Peter *et al.*, 1987; Habibi *et al.*, 1989), is in agreement with the finding that this analog has higher affinity to the pituitary binding sites than that of the native forms and other analogs. This superactive analog has been successfully applied to aquaculture to induce ovulation and spawning in various commercially important fishes, including Chinese carps (Lin *et al.*, 1988; Peter *et al.*, 1988), Chinese loach (Lin *et al.*, 1988), and sea bass (Almendras *et al.*, 1988).

The stimulation of GtH release from the pituitary gland by exogenous GnRH is a complex process influenced by many factors. The magnitude and duration of GtH release after the administration of different forms of GnRH and GnRH analog may be affected by the half-life of each form as well as the affinity to the pituitary receptors. Sherwood and Harvey (1986) used radioimmunoassay (RIA) to measure plasma mGnRH levels following a single intraperitoneal (ip) injection of synthetic mGnRH in goldfish. Their results indicate that the rate of clearance of mGnRH from the plasma is rapid, as half of the hormone present at the peak (4 min after injection) disappears 12 min later. This initial half disappearance time $T_{(1/2)i}$ of mGnRH in goldfish is slightly longer than that of mGnRH in mammalian species; in the rat the $T_{(1/2)i}$ after intravenous injection ranges from 3-10 min (Miyachi *et al.*, 1973; Redding and Schally, 1973; Swift and Crighton, 1979). Although the half-lives of some peptide hormones in circulation have been reported to be prolonged by binding to their specific carrier proteins (Baumann *et al.*, 1986, 1987; Cohen and Nissley, 1976; Hodgkinson *et al.*, 1989), mGnRH in human is bound nonspecifically with serum albumin only to a negligible extent (Tharandt *et al.*, 1979), which is reflected

in the short $T_{(1/2)i}$ of 2-7 min (Arimura *et al.*, 1974; Jeffecoate *et al.*, 1974; Pimstone *et al.*, 1977; Tharandt *et al.*, 1981). There is no information available concerning the serum metabolic clearance rate (MCR) and initial distribution volume of injected GnRH in teleost fishes.

Because of the direct innervation of the pituitary by GnRH fibers, the importance of endogenous circulating GnRH in goldfish is unclear. However, successful application of exogenous GnRH and GnRH analogs in inducing ovulation and spawning in many teleost species including goldfish (Almendras *et al.*, 1988; Carolsfeld *et al.*, 1988; Crim *et al.*, 1986; Lee *et al.*, 1987; Peter *et al.*, 1987), and the uptake into circulation of exogenous GnRH applied in different ways (Sherwood and Harvey, 1986) indicate that GnRH in circulation certainly is effective in stimulating GtH release from the pituitary.

In this thesis I studied in the goldfish the circulating forms of GnRH in goldfish, the serum clearance of intra-arterially (ia) injected ^{125}I-sGnRH and ^{125}I-sGnRH-A, the tissue uptake of ^{125}I-sGnRH-A, and the presence and characterization of a GnRH binding protein. The major findings in this thesis are arranged into five main chapters (chapter 2 to chapter 6). Chapter 2 and Chapter 3 describe the characterization and purification of a specific GnRH binding protein in goldfish serum. The circulating forms of GnRH in goldfish is investigated in Chapter 4. Chapter 5 outlines the seasonal changes of the GnRH binding protein and the effects of hormone treatment on the GnRH binding protein. Chapter 6 addresses the tissue uptake and serum clearance of injected GnRH; the possible involvement of GnRH binding protein in the metabolism of GnRH is also discussed. Finally, Chapter 7 summarizes and discusses the major findings of the thesis, and suggests some remaining problems for future studies.

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2. EVIDENCE FOR A GONADOTROPIN-RELEASING HORMONE (GNRH) BINDING PROTEIN IN GOLDFISH SERUM [1]

2.1 INTRODUCTION

In 1979, Tharandt *et al.* reported high capacity, low affinity, nonspecific albumin binding of gonadotropin-releasing hormone (GnRH, pGlu^1, His^2, Trp^3, Ser^4, Tyr^5, Gly^6, Leu^7, Arg^8, Pro^9, Gly^{10}-NH$_2$) and [D-Leu^6, Pro^9-NEt]-GnRH in human serum. Recently, Chan and Chaplin (1985) reported the binding of GnRH and [3-(2-naphthyl)-D-Ala^6]-GnRH (nafarelin acetate), a highly potent GnRH agonist, to plasma protein of normal human subjects, female rhesus monkeys and female rats (Chan and Chaplin, 1985). No specific binding of GnRH to mammalian serum protein has been reported. To date, there are no published data on the binding of GnRH to serum proteins in teleosts or other non-mammalian vertebrates.

The primary structure of a gonadotropin-releasing hormone in chum salmon has been determined to be [Trp^7, Leu^8]-GnRH (sGnRH) (Sherwood *et al.*, 1983). Studies using a variety of approaches have demonstrated sGnRH to be present in many species of teleost fish including goldfish (Breton *et al.*, 1984, 1986; Sherwood *et al.*, 1984; King and Millar, 1985; Powell *et al.*, 1986; Sherwood and Harvey, 1986; Sherwood and Lovejoy, 1989; Yu *et al*, 1988). Structure-activity studies on GnRH and sGnRH *in vivo* in goldfish demonstrated [D-Arg^6, Trp^7, Leu^8, Pro^9-NEt]-GnRH (sGnRH-A) to be a highly potent agonistic analog in stimulating gonadotropin release *in vivo* in goldfish (Peter *et al.*, 1985). Studies on the activity of a number of analogs of GnRH indicated that

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1. A version of this chapter has been published. Huang, Y. P. and Peter, R. E. 1988. Gen. Comp. Endocrinol. 69: 308-316.

sGnRH-A was the most active of all analogs tested in stimulating gonadotropin (GtH) release from perifused fragments of goldfish pituitary; sGnRH-A also had the highest affinity for the high affinity/low capacity binding sites in the goldfish pituitary (Habibi *et al.*, 1989). In the present study, the binding of sGnRH and its superactive analog sGnRH-A to goldfish serum protein was investigated, using ^{125}I-sGnRH-A and ^{125}I-sGnRH as labeled ligands. The results of our studies provide evidence for the existence and properties of a specific GnRH binding protein in goldfish serum.

2.2 MATERIALS AND METHODS

Goldfish, *Carassius auratus*, common and comet varieties, were purchased from Grassyforks Fisheries Co., Martinsville, Indiana. The fish were held in 96-liter flow through aquaria at 20±1 °C on a simulated natural (Edmonton) photoperiod for at least 4 weeks, and fed a commercial diet (EWOS).

The serum used in the studies was collected between August and October from a combination of males and females that were sexually regressed or in early stages of gonadal recrudescence (Note it was not possible to externally sex the fish, necessitating use of mixed sexes). Fish were anesthetized in 0.05% tricaine methanesulfonate, and blood was collected by puncture of the caudal vasculature with a 25-gauge needle attached to a 1-ml syringe. The blood was allowed to clot at 4 °C for several hours, and the serum collected following centrifugation at 13,000g for 5 min. The serum was pooled and stored at -20 °C or used fresh.

Salmon GnRH and sGnRH-A were synthesized and kindly provided by J. Rivier and W. W. Vale (The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, San Diego, CA).

The iodination procedure and the specific activity determination of the ^{125}I-sGnRH were described previously (Yu *et al.*, 1987). Salmon GnRH-A was iodinated using a modified chloramine-T method as described previously (Habibi *et al.*, 1987). Determination of specific activity of the ^{125}I-sGnRH-A was carried out on a routine basis by a self-displacement radioimmunoassay; the mass-radioactivity relationship was estimated at the 50% binding level from the displacement curve obtained by incubating sGnRH-A antiserum with increasing concentrations of unlabeled sGnRH-A or ^{125}I-sGnRH-A. Specific activity of ^{125}I-sGnRH-A was 1,100-1,300 μCi/μg.

Bound peptide was separated from free peptide by gel filtration with Sephadex G-50

mini-columns. An elution buffer containing of 10 mM Tris-HCl, 1 mM dithiothretol and 0.5% bovine serum albumin (BSA), pH7.6, was used in all binding assays. Mini-columns. 1.3 x 3.0 cm, of Sephadex G-50 fine presoaked in Tris-HCl buffer, were saturated with BSA by eluting 0.5 ml of 5% BSA dissolved in Tris-HCl buffer through with 4 ml of elution buffer; freshly made mini-columns were kept at 4 °C before use.

Prior to incubation with peptides, serum samples were initially diluted 1 : 10 with Tris-HCl buffer containing 0.5% BSA; exceptions to this procedure are outlined in the Results. Fifty µl aliquots of diluted serum were pipetted into 12 x 75 mm glass tubes, along with about 30,000 CPM tracer in 200 µl Tris-HCl buffer and various concentrations of unlabeled peptides in Tris-HCl to give a final incubation volume of 600 µl (1 : 120 final dilution of the goldfish serum). Incubation was done at 4°, unless otherwise specified, for different period of time as indicated in the Results. Following incubation, a 500 µl aliquot of the incubate was pipetted onto a mini-column of Sephadex G-50; the eluate contained no radioactivity and was discarded. Large proteins, with bound sGnRH or sGnRH-A, were eluted in the void volume of the mini-column with 1.7 ml ice cold Tris-HCl buffer, and collected as one fraction within a period of 3.5 min. The radioactivity of the 1.7 ml eluate (the protein peak) was measured in a gamma spectrometer with a counting efficiency of 75% for ^{125}I. Specific binding was calculated by subtracting the radioactivity measured in the presence of 10^{-5} M unlabeled sGnRH-A. Binding of ^{125}I-sGnRH-A to BSA in the absence of goldfish serum was less than 5%. In the experiment where ^{125}I-sGnRH was used, the bound data were calculated following the application of a correction factor for the maximum immunoreactivity of the ^{125}-I labeled sGnRH preparation (45% of the radioactivity was immunoprecipitable by an excess 1 : 3,000 final dilution of antiserum PBL-49; Yu *et al.*, 1987). Since about 90% radioactivity of the ^{125}I-sGnRH-A preparation was immunoactive (precipitable by excess amount of antiserum), no correction was applied for the calculation of bound ^{125}I-sGnRH-A. All binding assays were carried

out in triplicate.

An aliquot of each pool of goldfish serum was used for protein determination according to Bradford's method, using BSA as standards (Bradford, 1976).

A non-linear model fitting computerized program (LIGAND) was used for Scatchard analysis and determination of best fit in displacement curve fitting (Fig. 2.4, insets). Where appropriate, statistical comparisons were made using Student's *t* tests.

2.3 RESULTS

Elution Profile of Sephadex G-50 Mini-column

A Sephadex G-50 mini-column was able to separate macromolecular proteins from free GnRHs (Fig. 2.1). Blue Dextran was eluted in the void volume as a single peak while free cold sGnRH-A (measured by sGnRH-A RIA) was retarded and eluted later as the second peak (Fig. 2.1A). ^{125}I-sGnRH-A bound to the binding protein was eluted in the void volume, similar to Blue Dextran; free ^{125}I-sGnRH-A was eluted later similar to free cold sGnRH-A (Fig. 2.1B).

Equilibrium Binding of sGnRH-A

Time-dependence of specific binding of ^{125}I-sGnRH-A to 1 : 120 dilution of goldfish serum was studied during incubation at 4 °C for a total period of 24 hours (Fig. 2.2). Maximum specific binding of the radioligand was achieved after 2 hours of incubation, and both total binding and specific binding were stable for the following 22 hours. Therefore, a 10 hour preincubation was used as standard equilibrium condition for the subsequent studies. In a separate experiment, incubation at 18-20 °C for 24 hours resulted in a slightly lower but not significantly different ($P>0.05$) binding than at 4 °C (data not shown).

Serum Concentration and sGnRH-A Binding

Goldfish serum diluted from 1 : 3 to 1 : 300 (final dilution) was used to study the correlation between serum concentration and ^{125}I-sGnRH-A binding (Fig. 2.3). Less than 5% of the total added radioligand was found to bind nonspecifically to BSA in the absence of diluted goldfish serum. The binding of ^{125}I-sGnRH-A to the diluted serum was concentration-dependent. At a 1 : 3 dilution, about 80% of the added ^{125}I-sGnRH-A was found in the bound form, while at 1 : 300 dilution, the binding was still significantly higher

($P<0.01$) than in the absence of goldfish serum. The binding of native ^{125}I-sGnRH to the diluted serum was lower than the analog, but the binding was also concentration-dependent (Fig. 3.3, inset).

Dissociation Curve

Goldfish serum diluted at 1 : 120 was preincubated with about 30,000 CPM of ^{125}I-sGnRH-A for 10 hours at 4 °C, followed by addition of excess (10^{-5} M) unlabeled sGnRH-A. Bound was separated from free ^{125}I-sGnRH-A with mini-columns at various time intervals after addition of unlabeled hormone, to determine the time course of ^{125}I-sGnRH-A dissociation (Fig. 2.4). The binding of ^{125}I-sGnRH-A to goldfish serum was reversible, and dissociation was rapid. Notably, almost all of the ^{125}I-sGnRH-A bound to goldfish serum was displaced by excess unlabeled sGnRH-A. The binding of ^{125}I-sGnRH-A to BSA was not dissociable.

Displacement Curve

Dose-related displacement of the specifically bound ^{125}I-sGnRH-A by unlabeled hormone is shown in Fig. 2.5. Addition of unlabeled sGnRH-A at a concentration greater than 10^{-5} M resulted in total displacement of the ^{125}I-sGnRH-A bound specifically to goldfish serum. In a separate experiment, the ^{125}I-sGnRH-A bound to BSA (less than 5% of total) was found to be non-saturable and non-displaceable by 10^{-5} M unlabeled sGnRH-A, and, therefore is considered to be non-specific (data not shown). A non-linear model fitting computerized program (LIGAND) was used for Scatchard analysis and determination of best fit (Munson and Rodbard, 1980). The results indicate that sGnRH-A displacement can be most satisfactorily described by a one site model (Fig. 2.5, inset). This binding site is of low affinity ($Ka = 2.20 \times 10^{6}$ M^{-1}) and high capacity ($R = 3.56 \times 10^{-10}$ mol/mg serum protein). Addition of unlabeled sGnRH also resulted in a dose-dependent

displacement of ^{125}I-sGnRH-A bound to goldfish serum (Fig. 2.6); sGnRH has a lower affinity for the same binding site than sGnRH-A.

The specificity of GnRH binding site was also studied in a separate displacement experiment, including other peptide hormones. Addition of 10^{-6} or 10^{-5} M unlabeled melanocyte-stimulating hormone (α-MSH), ß-endophin, neurotensin or vasoactive intestinal peptide (VIP) resulted in essentially no displacement (Fig. 2.7). In another experiment, addition of 10^{-6} or 10^{-5} M somatostatin was also found to be ineffective in displacing the bound ^{125}I-sGnRH-A (data not shown).

Protein Nature of the Binding Component

When goldfish serum was chromatographed with Sephadex G-50-150 column, the serum binding component co-eluted with the Blue Dextran marker in the void volume. Incubation of goldfish serum with protease (Dispase, Boehringer) at 37 °C for 4 hours destroyed the the binding ability of the serum (data not shown). These results suggest that the GnRH macro-binding component is a serum protein.

2.4 DISCUSSION

Although there is some information available about GnRH binding proteins in mammalian serum (Tharandt *et al.*, 1979; Chan and Chaplin, 1985), this is the first study to attempt to quantify the binding affinity and capacity of such a binding protein in a vertebrate species. The data demonstrate that binding of sGnRH in goldfish serum is saturable, reversible and specific.

Dissociation of the ligand-protein complex is low in the mini-column separation system (Yoshimi and Lipsett, 1968). Although the binding affinity of the sGnRH binding protein in goldfish serum is low (see below), the use of a cooled mini-column, cooled elution buffer and very short elution time (less than 3.5 min) minimized the possible dissociation during the separation procedure. This was confirmed by the detection of 80% bound ^{125}I-sGnRH-A after gel filtration of 1 : 3 diluted goldfish serum preincubated with ^{125}I-sGnRH-A (Fig. 2.3).

Results obtained in the experiment studying the time course of binding (Fig. 2.2) demonstrated the stability of both the binding protein and the labeled ligand during the course of incubation; at the end of a 24 hour incubation at 4 °C both total binding and specific binding were essentially the same as at the end of a 2 hour incubation. Also, incubation at 18-20 °C for 24 hours resulted in a slightly lower, but not significantly different binding of ^{125}I-sGnRH-A or ^{125}I-sGnRH than at 4 °C (data not shown). There were no significant differences in binding ability between fresh and pre-frozen goldfish serum (Fig. 2.3); the freezing process *per se* apparently does not affect binding ability. The source of the GnRH binding protein in goldfish serum is unclear.

The observations based on displacement studies indicate that both sGnRH-A and native sGnRH compete for the same binding site in goldfish serum (Fig. 2.6); the binding site is of low affinity and high capacity (for sGnRH-A, $K_a = 2.20 \times 10^6\ M^{-1}$; $R = 3.56 \times 10^{-10}$

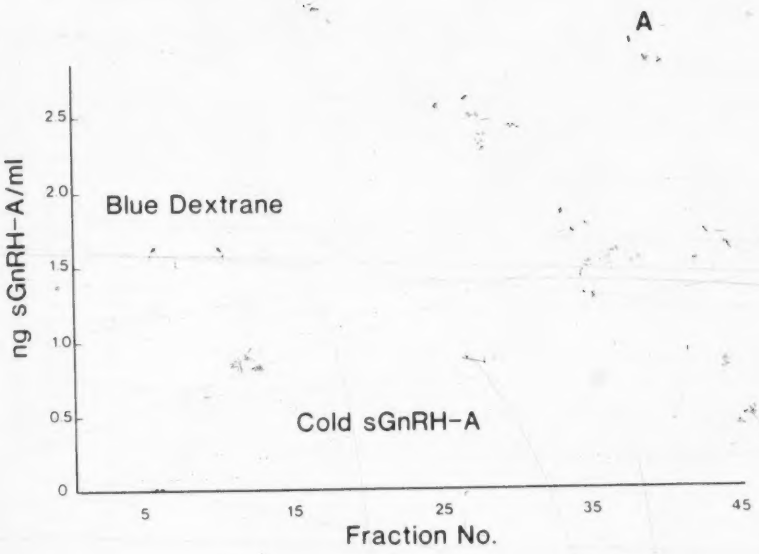
mol/mg serum protein). The instability of peptide-binding protein complex was demonstrated in the dissociation experiment, with a half time of dissociation of less than 2 min (Fig. 2.4); this confirms that the serum binding protein is of low affinity. In human serum, GnRH and [D-Leu^6, Pro^9-NEt]-GnRH bind to serum proteins and, although the binding was not quantified, the binding site is suggested to be of low affinity and high capacity (Tharandt *et al.*, 1979).

The sGnRH receptors of the goldfish pituitary have both high affinity/low capacity (K_{a1} = 2.28±0.29 x 10^{10} M^{-1}, R = 8.8 x 10^{-15} mol/mg membrane protein), and low affinity/high capacity (K_{a2} = 2.43 x 10^7 M^{-1}, R = 4.9 x 10^{-12} mol/mg membrane protein; Habibi *et al.*, 1987). The GnRH receptors in the pituitary have a slow dissociation rate (half dissociation time is 68.6 min for the rapid component and 451.5 min for the slow component (Habibi *et al.*, 1987). Although the sGnRH binding protein in serum is more abundant than the high-affinity binding site in the pituitary (3.56 x 10^{-10} mol/mg serum protein vs 8.8 x 10^{-15} mol/mg membrane protein), the lower affinity and the relative rapid dissociation of the hormone-protein complex in the circulation would favor transfer of sGnRH into the pituitary and, eventually, full occupancy of pituitary receptor sites.

Based on the characteristics of the serum binding protein in comparison with those of pituitary GnRH receptors in goldfish, I conclude that the serum binding proteins would not limit, but rather enhance, the uptake of any circulating endogenous or exogenous sGnRH into the pituitary. The binding protein would prevent clearance of sGnRH or sGnRH-A by the kidneys and protect sGnRH or sGnRH-A from degradation by serum enzymes, thereby maintaining any endogenous or exogenous sGnRH or sGnRH-A in circulation, and allow more opportunity to bind to pituitary receptors. The binding protein is itself not a degradative enzyme as binding of ^{125}I-sGnRH-A or ^{125}I-sGnRH was stable after incubation for 24 hr at both 4 °C and 20 °C; moreover, the level of binding was similar at both temperatures. It is of interest to note that sGnRH-A, which has a higher biological

activity and a longer duration of action than native sGnRH *in vivo* (Peter et al., 1985), has a greater binding affinity to the serum than native sGnRH (Figs. 2.3 and 2.6). In future studies it will be of importance to correlate the *in vivo* turnover rate of both sGnRH-A and sGnRH with their respective binding to serum proteins in the goldfish.

Fig. 2.1. Elution profiles of Sephadex G-50 mini-columns. Each fraction contains 0.19 ml eluate with the exception of fraction #1 which contains 0.50 ml. Values of cold sGnRH-A in each fraction were measured by sGnRH-A RIA (A). Bound ^{125}I-sGnRH-A was coeluted with Blue Dextran in the void volume, while free tracer was coeluted with cold sGnRH-A (B).


A
ng sGnRH-A/ml
2.5
2.0
1.5
1.0
0.5
0
Blue Dextrane
Cold sGnRH-A
5
15
25
35
45
Fraction No.

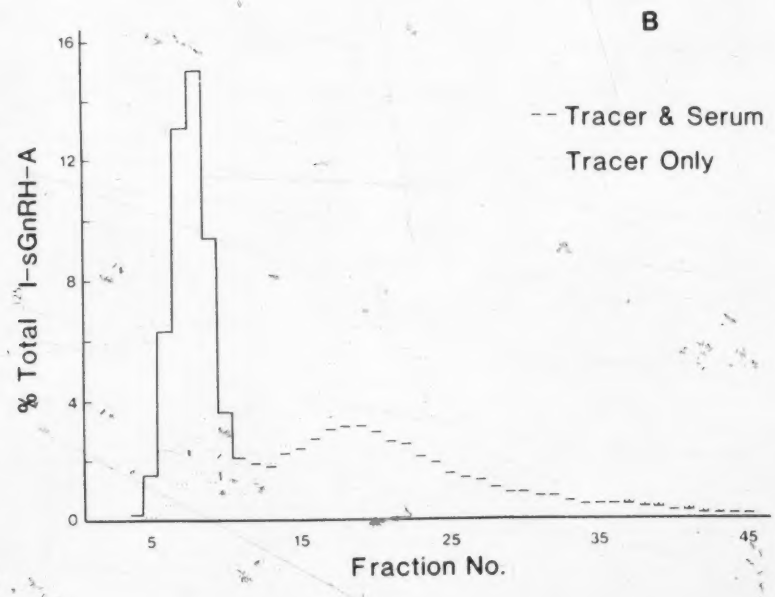

B
% Total 125I-sGnRH-A
16
12
8
4
0
-- Tracer & Serum
Tracer Only
5
15
25
35
45
Fraction No.

Fig. 2.2. Time dependence of ^{125}I-sGnRH-A binding to diluted goldfish serum (1 : 120). Data show fraction bound (B/T, percentage of total counts). Values of specific binding were determined by subtracting the nonsaturable binding in the presence of 10^{-5} M cold sGnRH-A from the total binding. Values are mean±SEM of triplicate determinations.

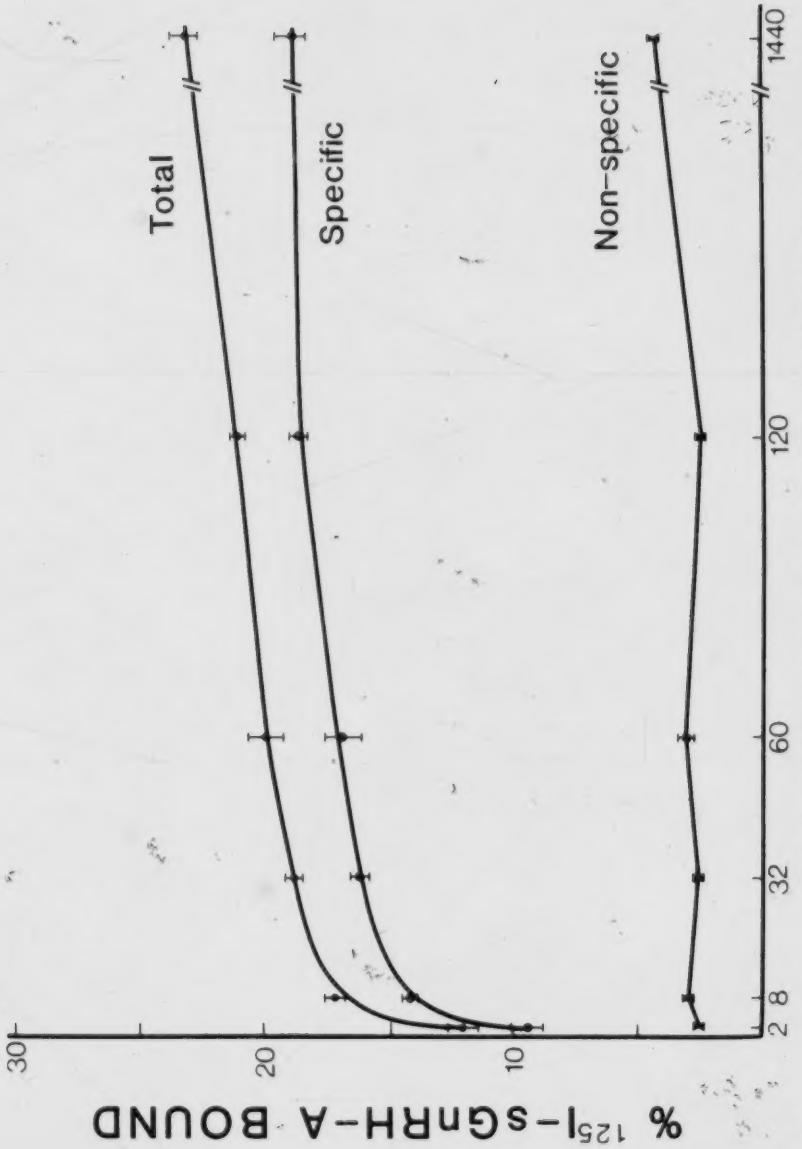

% 125I-sGnRH-A BOUND
30
20
10
2 8
32
60
120
1440
INCUBATION TIME (MINUTE)
Total
Specific
Non-specific

Fig. 2.3. Equilibrium binding (total) of ^{125}I-sGnRH-A to increasing concentrations of fresh or prefrozen goldfish serum (diluted from 1 : 300 to 1 : 3). Inset shows the equilibrium binding of ^{125}I-sGnRH to increasing concentrations of prefrozen goldfish serum diluted from 1 : 120 to 1 : 12. Values are mean±SEM of triplicate determinations.

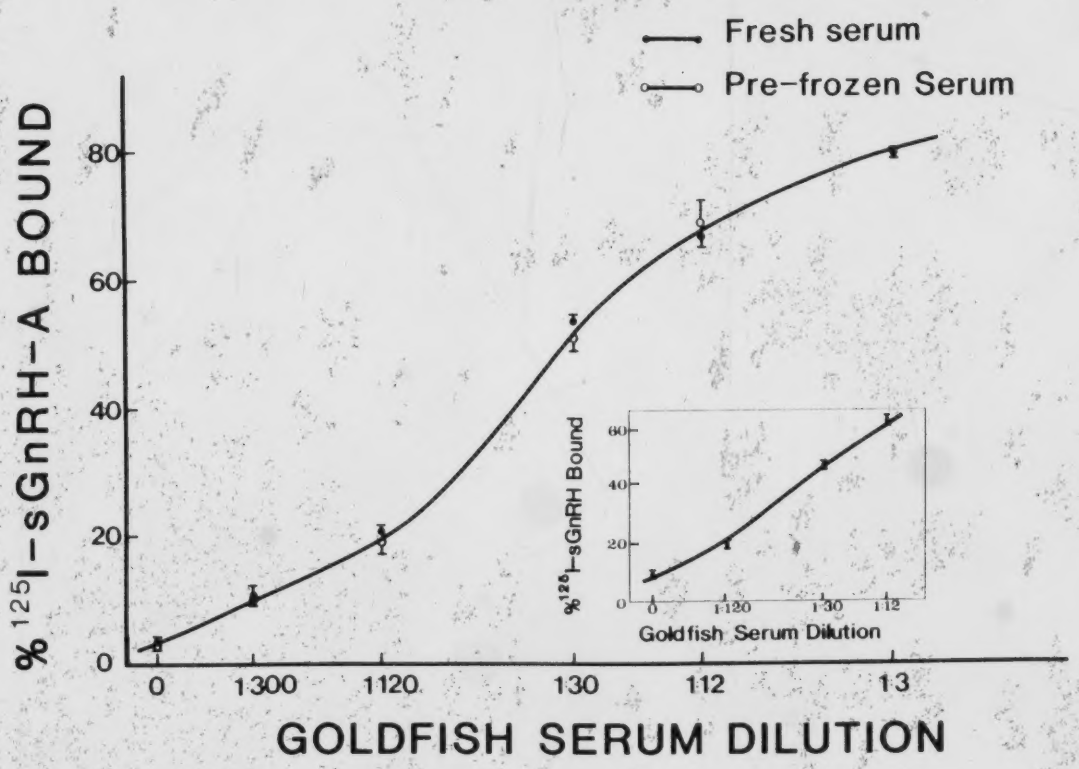

Fresh serum
Pre-frozen Serum
% 125I-sGnRH-A BOUND
80
60
40
20
0
0
1:300
1:120
1:30
1:12
1:3
GOLDFISH SERUM DILUTION
% 125I-sGnRH Bound
60
40
20
0
0
1:120
1:30
1:12
Goldfish Serum Dilution

Fig. 2.4. Dissociation curve of ^{125}I-sGnRH-A bound to diluted goldfish serum after addition of unlabeled sGnRH-A (10^{-5} M). Prior to addition of the unlabeled sGnRH-A, diluted goldfish serum (1 : 120) was preincubated with ^{125}I-sGnRH-A for 10 hours at 4 °C. Control incubations were carried out separately in the absence of cold sGnRH-A until the end of the experiment; after 24 hours of incubation, the bound fraction of ^{125}I-sGnRH-A was 26.9±2.0% of total, essentially the same as at time of addition of unlabeled sGnRH-A for the dissociation rate (27.8±1.7% of total). The binding of ^{125}I-sGnRH-A to BSA was not dissociable. Values are mean±SEM (n = 3).

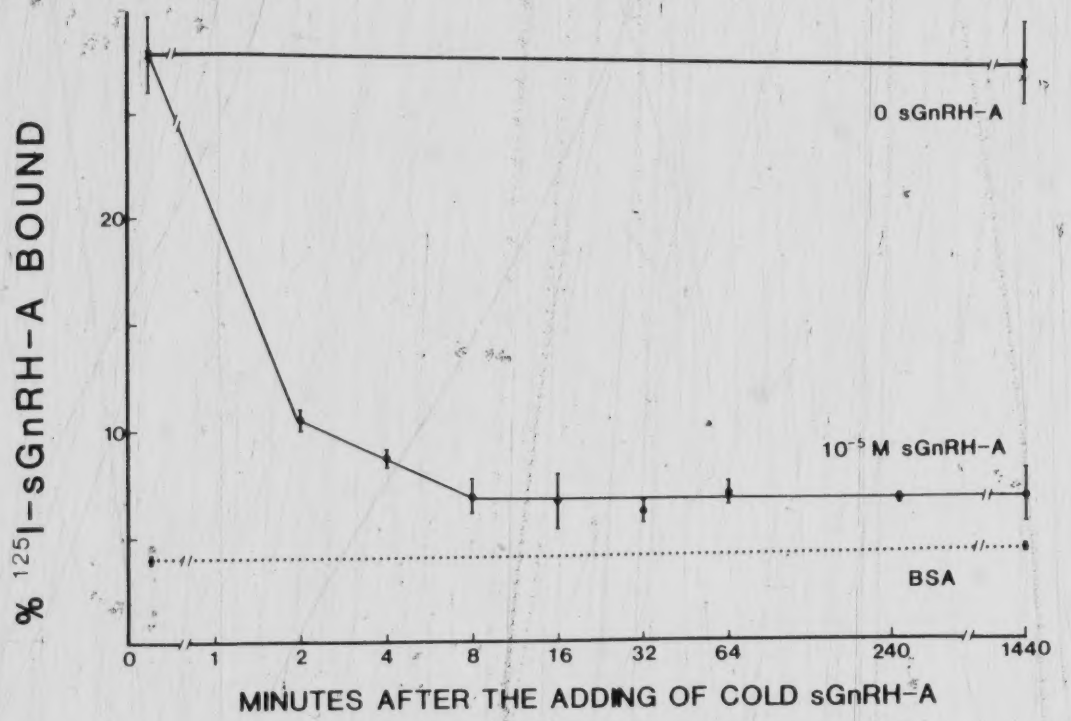

% 125I-sGnRH-A BOUND
20
10
0 sGnRH-A
10-5 M sGnRH-A
BSA
0
1
2
4
8
16
32
64
240
1440
MINUTES AFTER THE ADDING OF COLD sGnRH-A

Fig. 2.5. Displacement of ^{125}I-sGnRH-A bound to goldfish serum (1 : 120 dilution) by increasing concentrations of unlabeled sGnRH-A. Values (mean±SEM) represent specific binding (bound/total) determined by subtraction of nonsaturable binding in the presence of excess (3.3×10^{-5} M) unlabeled sGnRH-A. Results were obtained in 6 experiments carried out in triplicate. Insets show the best curve fit through the data points (A) and Scatchard plot of the displacement data (B) analyzed by a computerized curve-fitting program (LIGAND).

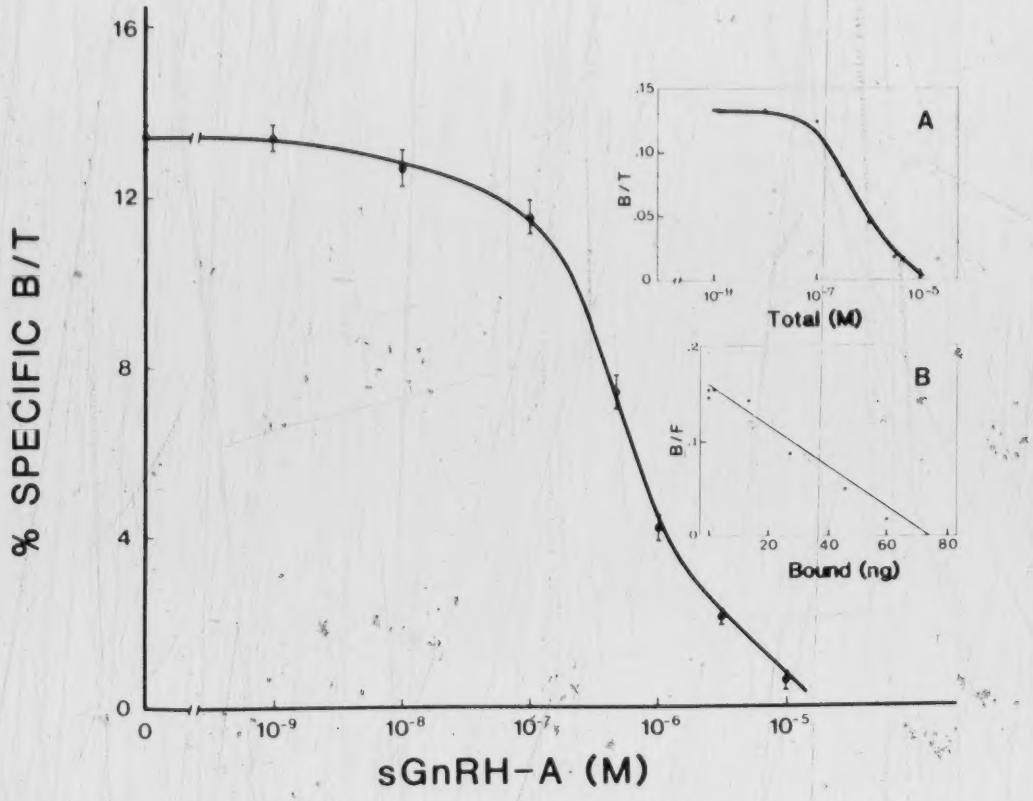

% SPECIFIC B/T
16
12
8
4
0
0
10-9
10-8
10-7
10-6
10-5
sGnRH-A (M)
A
B/T
.15
.10
.05
0
10-11
10-7
10-5
Total (M)
B
B/F
.2
.1
0
20
40
60
80
Bound (ng)

Fig. 2.6. Displacement of ^{125}I-sGnRH-A bound specifically to diluted goldfish serum (1 : 120 dilution) by unlabeled sGnRH-A or sGnRH. Values are mean±SEM of triplicates.

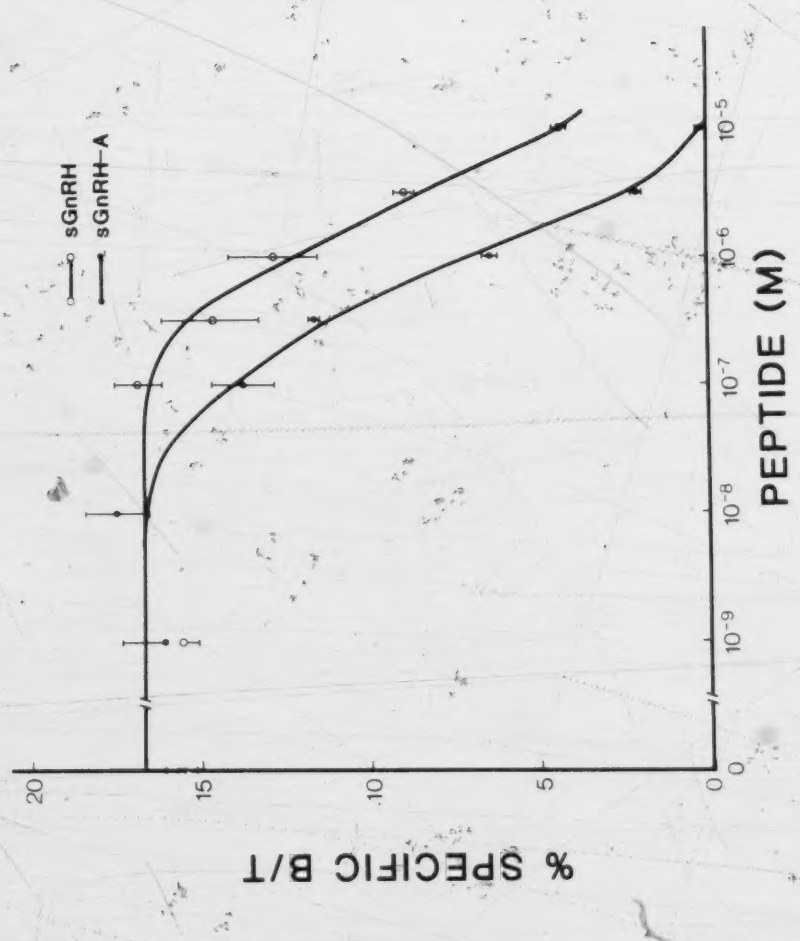
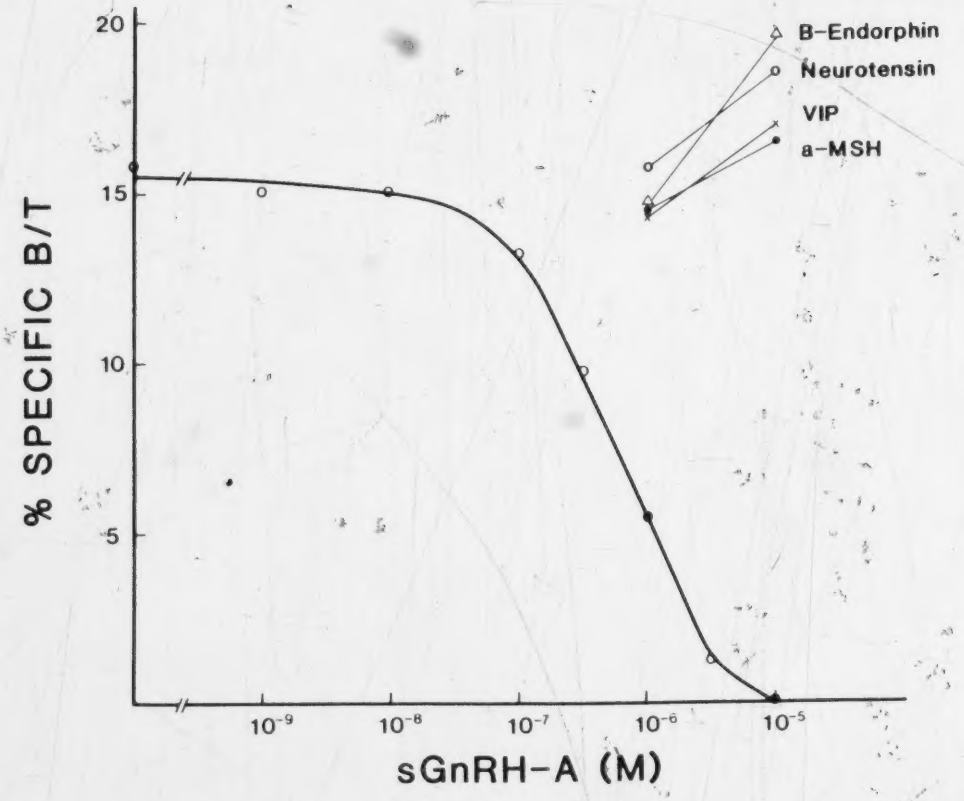

% SPECIFIC B/T
20
15
10
5
0
0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
PEPTIDE (M)
sGnRH
sGnRH-A

Fig. 2.7. Displacement of ^{125}I-sGnRH-A bound specifically to diluted goldfish serum (1 : 120 dilution) by 10^{-6} M and 10^{-5} M of ß-endophin, neurotensin, vasoactive intestinal peptide (VIP), and melanocyte-stimulating hormone (α-MSH), compared to various concentrations of unlabeled sGnRH-A. Values (mean of triplicates) are percentages of total counts.


20
15
10
5
% SPECIFIC B/T
B-Endorphin
Neurotensin
VIP
a-MSH
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
sGnRH-A (M)

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3. ISOLATION AND CHARACTERIZATION OF A GNRH BINDING PROTEIN FROM GOLDFISH SERUM

3.1 INTRODUCTION

We have previously reported the affinity characteristics of a gonadotropin-releasing hormone (GnRH) binding protein (BP) in goldfish serum (Huang and Peter, 1988). In addition, we detected [His^5, Trp^7, Tyr^8]-GnRH (cGnRH-II) and [Trp^7, Leu^8]-GnRH (sGnRH) in goldfish serum using radioimmunoassays (RIA) with different GnRH antisera after high pressure liquid chromatography (HPLC) separation (Peter *et al.*, 1990). Non-specific binding of mammalian GnRH (mGnRH) and its superactive analogue, [D-Leu^6, Pro^9]-mGnRH to human serum albumin has been reported (Tharandt *et al.*, 1979). In the present study, a specific GnRH binding protein was isolated from goldfish serum, using native polyacrylamide gel electrophoresis. The binding protein was further purified by HPLC to near homogeneity. Some properties of the GnRH binding protein were also investigated.

3.2 MATERIALS AND METHODS

Source and Maintenance of Fish

Goldfish, *Carassius auratus*, of the common or comet varieties were purchased from Grassyforks Fisheries Ltd., Martinsville, IN. All fish were maintained for a minimum of 28 days in 96-liter flow-through aquaria, under a daily cycle of 16 hours light and 8 hours dark at 20 ± 1 °C, prior to experimental treatment. At random times during the photophase the fish were fed a commercial chow (Ewos) to excess.

The serum used in the studies was collected from a combination of male and female goldfish at different seasons throughout the year. Fish were anesthetized in 0.05% tricaine methanesulfonate, and blood was collected by puncture of the caudal vasculature with a 25-gauge needle attached to a 1-ml syringe. The blood was allowed to clot at 4 °C for several hours, and the serum collected following centrifugation at 13,000g for 5 min. The serum was pooled and stored at -20 °C until use. No qualitative change of characteristics of serum BP was noticed from pool to pool.

Chemical Reagents

Synthetic sGnRH, [D-Arg^6, Pro^9-NEt]-sGnRH (sGnRH-A) and [D-Lys^6, Pro^9-NEt]-sGnRH (sGnRH-L) were synthesized and kindly provided by J. Rivier and W. W. Vale (The Clayton Foundation Laboratories for Peptide Biology, the Salk Institute, San Diego, CA); disuccinimidyl suberate (DSS) was purchased from Boehringer Mannheim Canada, Dorval, Que; HPLC grade trifluoroacetic acid (TFA) was purchased from Pierce Chemical, Rockford, IL; and HPLC grade acetonitrile from Fisher Scientific, Fairlawn, NJ. Double-distilled water was purified by passing it through a Milli-Q water purification system.

(Millipore, Bedfold, MA). Konica A-7A X-ray films for autoradiography were from Konica.

Preparative Polyacrylamide Gel Electrophoresis

The goldfish serum proteins were separated by using native polyacrylamide gel electrophoresis (PAGE). The purpose of this step was to isolate the non-denatured GnRH binding protein (GnRH-BP) from the complex protein mixture. Briefly, native PAGE was performed on 8% slab gels (12 X 17 cm) using a Tris borate buffer (6.3 g Tris base, 0.87g boric acid per liter, pH 9.0) as running buffer. Electrophoresis of samples was accomplished in 5-6 hours at room temperature with constant current of 15 mA / gel. Samples were prepared for electrophoresis by adding one part of sample buffer (50% glycerol, 50% Tris borate buffer with bromophenol blue added as a tracking dye) to three parts of goldfish serum. After electrophoresis, one lane of each gel was stained with 0.025% Coomassie Blue G-250 in 70% perchloric acid and destained in 7% acetic acid to give the electrophoregram of the native gel (Fig. 3.1), and the rest of the gel was cut (without stain) from bottom to top into fractions of 0.5 cm-slice. The proteins in each gel fraction were extracted with either 10 mM Tris-HCl, pH 7.4 or 40 mM phosphate buffer, pH 7.4. Ligand binding test was performed to screen the gel fractions containing the GnRH-BP. The protein content of each fraction was determined according to Bradford's method, using bovine serum albumin (BSA) as standards (Bradford, 1976).

Ligand Binding Assay for GnRH Binding Protein

Ligand binding activity of each fraction was determined using ^{125}I-sGnRH-A as previously described (Huang and Peter, 1988). In brief, protein extracts were incubated

for 10 hours at 4 °C with approximately 30,000 cpm of ^{125}I-sGnRH-A in a 600 µl final volume of 10 mM Tris-HCl buffer, pH 7.4, containing 0.5% BSA. To separate bound and free tracer, a 500-µl aliquot of the incubate was pipetted onto a mini-column of Sephadex G-50, and the radioactivity eluted in the early peak (the ligand-protein complex) was collected into one 1.7 ml fraction and measured in a gamma scintillation counter (Model MS 4/600, Micromedic System Inc. Ltd., Horsham, PA) with counting efficiency of about 75% for ^{125}I.

Analytical Polyacrylamide Gel Electrophoresis

Electrophoresis on 12% polyacrylamide slab gels (Mini-Protean II System, BioRad) containing 0.1% sodium dodecyl sulfate (SDS) under reducing condition (1% 2-mercaptoethanol) was conducted at room temperature, as described by Laemmli (1970). After electrophoresis, the gel was either blotted to a nitrocellulose sheet for glycoprotein stain or stained for 1 hour with 0.01% Coomassie Blue R-250 in 40% methanol-10% acetic acid, and destained with 40% methanol-10% acetic acid. Subsequent silver staining of the gels was done by the method of Morrissey (1981).

Affinity Labeling

Affinity cross-linking of ^{125}I-sGnRH-L to GnRH-BP was carried out using the methods described by Massague and Czech (1982) with modifications. Diluted goldfish serum or isolated GnRH-BP was incubated with approximately 200,000 cpm of ^{125}I-sGnRH-L in the presence or absence of 10^{-3} M unlabeled sGnRH-L in a final volume of 90 µl of 40 mM phosphate buffer, pH 7.4, for 10 hours at 4 °C. At the end of incubation, 10 µl of 5 mM DSS, prepared freshly in dimethylsulfoxide, was added to the incubate to give

a final concentration of 0.5 mM DSS. After 5 min at room temperature, the cross-linking reaction was quenched by the addition of 10 µl of 1.0 M Tris-HCl, pH 7.4. The solution was mixed in a 1 to 2 ratio with sample buffer containing 2% SDS, 10% glycerol and 5% 2-Mercaptoethanol in Tris-HCl, pH 6.8. All samples were boiled for 5 min prior to loading onto the analytical gel for electrophoresis. A mixture of low molecular weight protein standards for gel electrophoresis was purchased from BioRad (BioRad Lab, Richmond, CA): phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 43,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400. Autoradiograms were made by exposing Konica A-7A X-ray film to the dried gel using DuPon Cronex intensifying screens (DuPont, Wilmington, DE) for 1-2 days at -70 °C.

Glycoprotein Stain

The isolated binding protein fractions were electrophoresed on analytical gel as described above. The gels were electroblotted for 2 hours at 150 V onto nitrocellulose sheets following the method of Towbin *et al* (1979) and using a BioRad Mini Trans-Blot Electrophoretic Transfer Cell filled with 25 mM Tris, 192 mM glycine and 20% methanol. The enzymatic affinity staining was performed using biotin labeled Con-A (Sigma Chemical Co., St. Louis, MO) and Vectastain ABC kit (Vector Lab Inc., Burlingame, CA).

Further Purification of the Binding Protein by HPLC

The HPLC instrument consisted of a Varian Vista Series 5000 liquid chromatograph (Varian, Walnut Creek, CA) combined with a Varian CS 401 data system and coupled to a Varian UV-50 variable-wavelength spectrometer. A Bio-Gel TSK-Phenyl-5-PW column was used (75 x 4.6 mm I. D., BioRad Lab, Richmond, CA). The partially purified

GnRH-BP from the preparative gel electrophoresis was directly injected into the hydrophobic interaction column. The solvent A was 0.1% TFA in water (pH 2.5) and solvent B was 0.1% TFA in acetonitrile. A linear AB gradient of 0.5% B/min was used with a flow rate of 1 ml/min. Fractions were collected at 1 min (1 ml) intervals; proteins were detected at a wavelength of 210 nm. The radioactivity level in each fraction was measured in the gamma scintillation counter (model MS 4/600, Micromedic System Inc. Ltd, Horsham, PA).

Statistics

Mann-Whitney U test was used for the test of specificity of the isolated GnRH-BP. A version of ALLFIT, a computerized four-parameter logistic curve-fitting program, was used for determination of ED_{50} values of the dose dependent displacement data (De Lean *et al.*, 1978).

3.3 RESULTS

Electrophoregram of preparative gel in Fig. 3.1 shows that the GnRH binding protein is not a major component of goldfish serum proteins. This native gel was not for molecular weight measurement; however, it was for the purpose of separating the binding protein from the majority of serum proteins without being denatured. The unstained portion of the native gel was cut from bottom to top into fractions of 0.5 cm-slice. Proteins in each fraction were extracted in 10 mM Tris-HCl buffer, pH 7.4, or 40 mM phosphate buffer pH 7.4 after homogenization of the gel fractions. Fig. 3.2 shows the protein content of each fraction measured by Braford's method and the binding ability of each fraction tested in the ligand binding assay. Data in Fig. 3.2 confirm that the GnRH binding ability of goldfish serum is due to a single component of the serum proteins (in fraction C), and the concentration of this binding protein relative to other serum proteins is low. Because of the "smiling effects" on the gel and the method of cutting, a small portion of the GnRH-BP band was cut into adjacent fractions (fractions B and D).

Figs. 3.3 and 3.4 show the results of affinity labeling of the GnRH-BP to ^{125}I-sGnRH-L, using different doses of cross-linking reagent DSS. When ^{125}I-sGnRH-L was incubated with isolated GnRH-BP (fraction C), cross-linked by different doses of DSS and analyzed by SDS-PAGE under reducing condition, a single major band with molecular weight of about 40 KD was identified (Fig. 3.3). The affinity labeling of GnRH-BP with ^{125}I-sGnRH-L by the cross-linking reagent DSS was dose dependent (Fig. 3.3), and was also time and temperature dependent (data not shown). The affinity labeling technique was used to visualize the binding protein (Fig. 3.4). Diluted goldfish serum or isolated GnRH-BP (fraction C) was incubated with ^{125}I-sGnRH-L and treated with or without 0.5 mM DSS. With the treatment of DSS, a major band with molecular weight of about 40 KD was visualized by autoradiography (Fig. 3.4, lanes 5 and 7). Unlabeled sGnRH-L totally

abolished the radioactivity from the 40 KD band (Fig. 3.4, lane 6). As shown in Fig. 3.4, some of the radioactivity did not penetrate into the gel under reducing conditions, and this may suggest a formation of protein aggregates.

The isolated GnRH-BP was further purified by high pressure liquid chromatography. Fraction C (containing the majority of GnRH-BP and also some unknown proteins) was preincubated with ^{125}I-sGnRH-L and treated with the cross-linking reagent DSS; the cross-linking reaction was quenched by 1.0 M Tris-HCl, pH 7.4. After affinity labeling, the product was loaded into a hydrophobic interaction column (Bio-Gel TSK-Phenyl-5-PW) and four major peaks were well separated (Fig. 3.5). As shown in Fig. 3.5, the radioactive peak was coeluted with the first protein peak, indicating that the first protein peak was affinity-labeled and represented the GnRH-BP; the remaining three peaks are unknown proteins. In subsequent runs of unlabeled fraction C on the hydrophobic interaction column, the first protein peak was collected and analyzed by the analytical gel for comparison to the proteins in fraction C (Fig. 3.6). The isolated GnRH-BP (fraction C) and the further purified GnRH-BP (HPLC peak 1) were run on a SDS gel (12% acrylamide) under reducing condition. Electrophoregrams in Fig 3.6. show that fraction C contains four major bands and a few minor bands, and the 40 KD GnRH-BP is one of the major bands (Fig. 3.6, lanes 3 and 5). On the other hand, the HPLC peak 1 contains only one major band of 40 KD (Fig. 3.6, lanes 2 and 4). It is noted that the GnRH-BP collected from HPLC peak 1 (Fig. 3.5) exhibits a very close electrophoretic mobility (40 KD) as compared to the results of the autoradiogram (compare Fig. 3.4 and Fig. 3.6).

The specificity of the isolated BP was shown in Fig. 3.7. Among the five known natural forms of GnRH, only sGnRH and cGnRH-II could displace the ^{125}I-sGnRH-A from the BP, whereas mGnRH, [Gln^8]-GnRH (cGnRH-I), [Tyr^3, Leu^5, Glu^6, Trp^7, Lys^8]-GnRH (lGnRH) did not cause displacement at a concentration of 10^{-5} M. The two superactive analogs tested, sGnRH-A and sGnRH-L, were also effective in displacing the

tracer. Fig. 3.8 shows the dose dependent displacement of bound ^{125}I-sGnRH-A by unlabeled sGnRH, cGnRH-II, and sGnRH-A. All three forms of GnRH displaced the bound tracer from the binding protein, with ED_{50} of 4.95×10^{-6} M, 2.19×10^{-6} M, 4.21×10^{-7} M for cGnRH-II, sGnRH and sGnRH-A, respectively.

To test whether the GnRH-BP was a glycoprotein, the isolated GnRH-BP was run on the analytical SDS PAGE, electroblotted onto nitrocellulose sheets, and enzymatic affinity staining performed using biotin labeled Con-A and Vectastain ABC kit. The 40 KD GnRH-BP band was negatively stained, indicating that the GnRH-BP is not a glycoprotein (data not shown).

3.4 DISCUSSION

We have previously reported the presence of GnRH-BP in goldfish serum (Huang and Peter,1988). In the present study, isolation and purification of the GnRH-BP from goldfish serum were described for the first time.

In contrast to the reported albumin binding of mGnRH and mGnRH analogue in human serum (Tharandt *et al.*, 1979), we found that sGnRH and sGnRH-A did not bind with albumin or any other major protein component of goldfish serum. A single minor band of serum protein was responsible for the total binding of GnRH (Figs. 3.1 and 3.2). After the isolation using the preparative gel, the binding protein extract was tested for its specificity for different forms of GnRH. This binding protein recognized both sGnRH and cGnRH-II, which are the two native forms of GnRH in goldfish (Peter *et al.*, 1990; Yu *et al.*, 1988), but not mGnRH, cGnRH-I, or lGnRH (Fig. 3.7). The two superactive analogs tested, sGnRH-A and sGnRH-L, were also effective in displacing the bound tracer from the isolated GnRH-BP (Figs. 3.7 and 3.8). The calculated ED_{50} values for sGnRH and cGnRH-II were 2.19×10^{-6} M and 4.95×10^{-6} M, respectively. The calculated ED_{50} for sGnRH-A was 4.21×10^{-7} M, similar to the affinity for unextracted goldfish serum reported previously ($Ka = 2.28 \times 10^{-6}$ M^{-1}, or $Kd = 4.51 \times 10^{-7}$ M; Huang and Peter, 1988). The superactive analog had higher affinity than both native forms. The binding protein does not crossreact with other peptide hormones tested, such as α-MSH, ß-endorphin, neurotensin, VIP, and somatostatin-14 (Huang and Peter, 1988). This binding protein appears to fulfill the criteria of relatively high affinity, limited binding capacity, high specificity, and reversibility for biologically relevant binding components.

A two-step purification scheme for the GnRH-BP in goldfish serum was used in this study. The first step involves native PAGE where the binding protein was partially purified and the binding ability for GnRH conserved. Then the partially purified binding

protein was further purified by hydrophobic interaction chromatography with a HPLC system. Water/TFA/acetonitrile solvent was shown to be an effective medium for the purification of the GnRH-BP from other impurities, although the purified product from HPLC was denatured and its binding ability was lost (data not shown).

Affinity cross-linking techniques have proved to be highly useful for the identification and structure characterization of receptors and carrier proteins. The covalently linked radiolabeled complexes are stable under denaturing conditions, enabling estimation of molecular size by SDS-PAGE and autoradiography. The estimated molecular weight of the GnRH-BP was about 40 K dalton (Figs. 3.3. and 3.4.). Our data (Fig. 3.2) excluded the major components of plasma proteins, such as vitellogenin or albumin, as being responsible for GnRH binding in goldfish serum.

There are some problems inherent in studies that employ chemical cross-linking agents (Peters and Richards, 1977). Because DSS can covalently cross-link any two free amino groups in the proper orientation and within an 11 angstroms radius, nonspecific bands resulting from random protein-ligand interactions were consistently observed when ^{125}I-sGnRH-L was cross-linked with unpurified goldfish serum (Fig. 3.4. lane 5). However, in contrast to the strongly labeled 40 KD band, only the few major protein bands were weakly labeled as the result of this nonspecific cross-linking. When ^{125}I-sGnRH-L was cross-linked with partially purified GnRH-BP (fraction C), there was no obvious nonspecific band (Fig. 3.4 lane 7).

The finding of a GnRH-BP is the most recent addition to the growing number of peptide hormone carrier proteins. The list includes human corticotropin releasing factor (CRF-41) binding protein (Linton and Lowry, 1986; Linton *et al.*, 1988; Orth and Mount, 1987), human growth hormone binding protein (Baumann and Amburn, 1986; Baumann *et al.*, 1986, 1987; Herinton *et al.*, 1986; Leung *et al.*, 1987), and insulin-like growth factor (IGF) binding proteins (Binoux *et al.*, 1982; Borsi *et al.*, 1982; Cohen *and*

Nissley, 1976; Hintz *et al.*, 1981; Hordgkinson *et al.*, 1989; Kaufmann *et al.*, 1978; Martin *and* Baxter, 1986; Moses *et al.*, 1976, 1979; White *et al.*, 1981; Wilkins and D'Ercole, 1985). Human CRF binding protein is specific for human CRF-41; it does not bind to ACTH, GnRH, vasopressin or even ovine CRF-41. CRF binding protein is found in human, but not in sheep or rat (Linton *et al.*, 1988; Orth and Mount,1987). Growth hormone binding protein is found in human and rabbit blood, but not in rat blood (Baumann *et al.*, 1987; Leung *et al.*, 1987). IGF binding proteins are found in both human and rat serum (Moses *et al.*, 1976, 1979; White *et al.*, 1981). The specific GnRH-BP is present in goldfish serum (present study), but not in human plasma (Tharandt *et al.*, 1979).

The biological significance of a circulating GnRH binding protein is at present unknown. The biological function of other polypeptide hormone binding proteins is still not well defined. Baumann *et al.* (1987) proposed that the human growth hormone binding protein may serve as a circulating buffer for GH, or a reservoir of GH, and that it would tend to dampen the oscillations caused by episodic secretion of hGH. Rutanen *et al.* (1988) found that human endometrium secretes a specific IGF-binding protein which acts as an autocrine/paracrine inhibitor of IGF action in this tissue, and that the IGF-binding protein complexes in circulation are probably delivered from rather than to their target. Cohen and Nissley (1976) reported that IGF binding proteins prolong the half life of IGF in circulation. sGnRH-A has a longer serum half life than sGnRH, correlating with the demonstrated affinities to the isolated GnRH-BP (chapter 6); the serum half-life of sGnRH found in the studies (Chapter 6) is longer than the serum half-life reported for mGnRH in goldfish (Sherwood and Lovejoy, 1986). In addition, goldfish serum normally contains extractable sGnRH and cGnRH-II in pg/ml quantities (chapter 4). Thus, we hypothesize that one important function of goldfish serum GnRH-BP may be to modify the metabolism of circulating sGnRH and cGnRH-II by complex formation.

Fig. 3.1. Electrophoregram of goldfish serum on preparative gel (8% native polyacrylamide gel). Extraction of proteins from gel fractions was followed by measurement of protein content and ligand binding assay, using ^{125}I-[D-Arg^{6}, Pro^{9}-NEt]-sGnRH as tracer.

E
D
C
B
A
BP

Fig. 3.2. Results of ligand binding assay and protein content measurement for extracted protein fractions. For the binding assay, each fraction was diluted 1:12 (final dilution), and bound was separated from free ^{125}I-[D-Arg^{6}, Pro^{9}-NEt]-sGnRH by chromatography using minicolumns of Sephadex G-50. Representative fractions A to E are indicated (also see Fig. 3.1). Fraction C (the fraction containing the majority of GnRH-BP) was used for further purification with HPLC system.

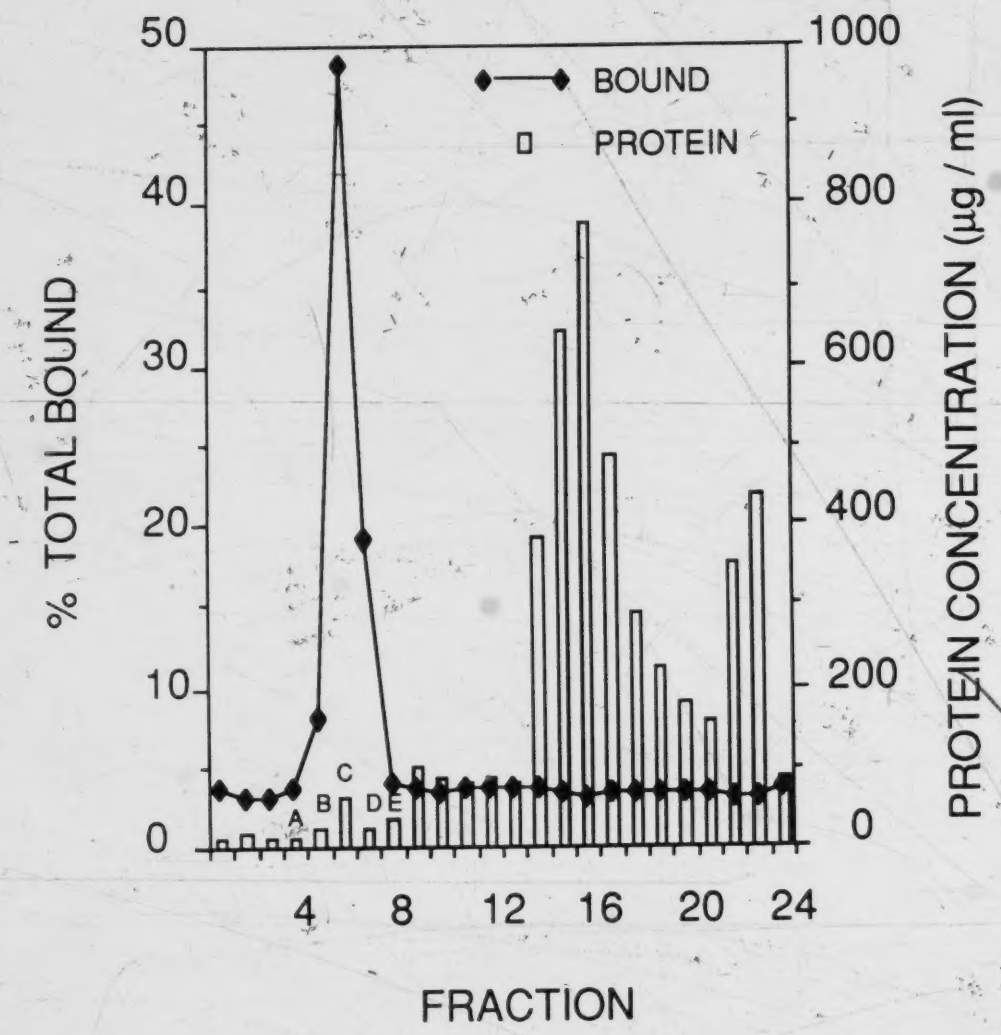

50
40
30
20
10
0
1000
800
600
400
200
0
BOUND
PROTEIN
% TOTAL BOUND
PROTEIN CONCENTRATION (µg / ml)
A
B
C
D
E
4
8
12
16
20
24
FRACTION

Fig. 3.3. Dose dependent affinity labeling of ^{125}I-[D-Lys^{6}, Pro^{9}-NEt]-sGnRH to the isolated GnRH-BP. Isolated binding protein (fraction C) was preincubated with ^{125}I-[D-Lys^{6}, Pro^{9}-NEt]-sGnRH and treated with different doses of disuccinimidyl suberate (open square: 0 mM, solid diamond: 0.15 mM, solid square: 0.5 mM). Following the affinity labeling, samples were run on a SDS-gel under reducing condition. Gel bands were cut at approximately 3 mm sections and counted for radioactivity. Bio-Rad prestained molecular weight standards were run on the same gel (molecular weight of standards 1 to 6: 17 KD, 27 KD, 39 KD, 50 KD, 75 KD, and 130 KD, respectively).

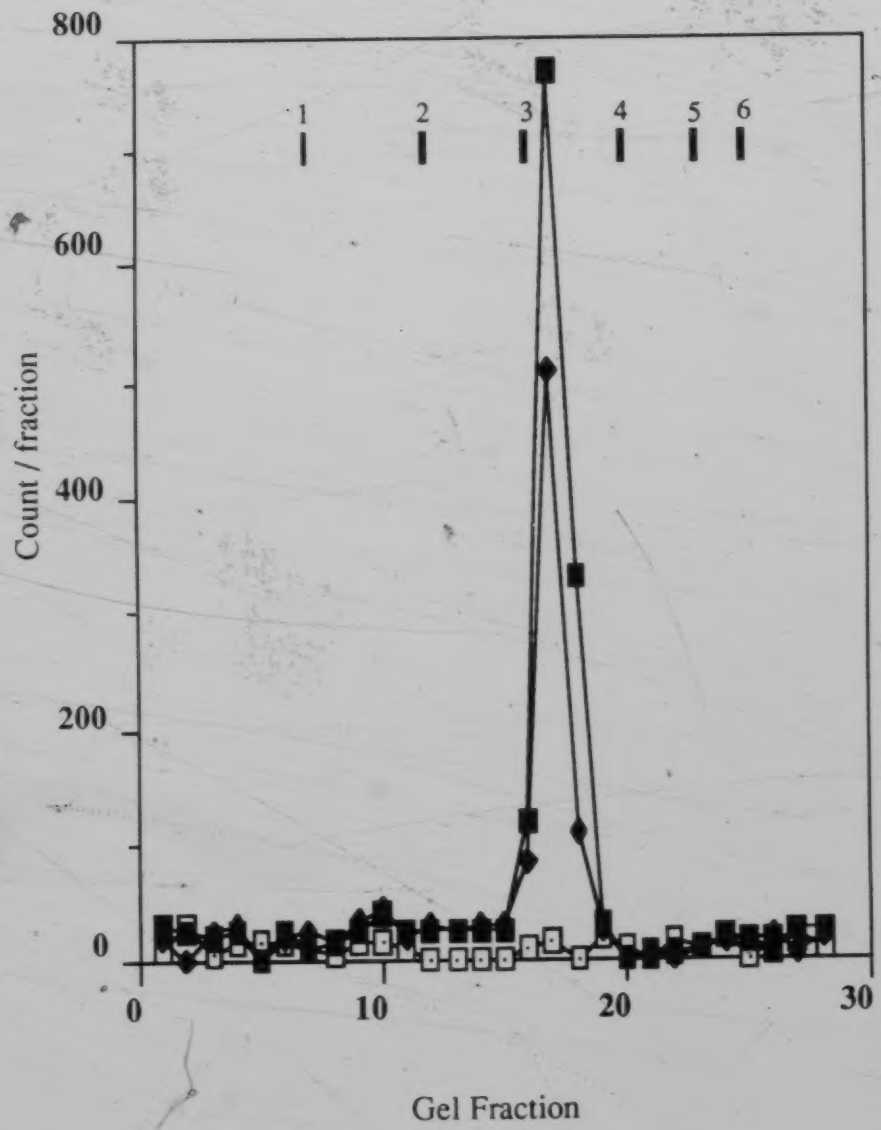

800
600
400
200
0
Count / fraction
1
2
3
4
5
6
0
10
20
30
Gel Fraction

Fig. 3.4. Visualization of the GnRH-BP by cross-linking to ^{125}I-[D-Lys^6, Pro^9-NEt]-sGnRH. Serum or isolated binding protein was incubated with ^{125}I-[D-Lys^6, Pro^9-NEt]-sGnRH and treated with or without 0.5 mM disuccinimidyl suberate. Lane 1 shows the low molecular weight standards (Bio-Rad). The other lanes show respectively: lane 2, the coomassie blue-stained pattern of serum proteins incubated with tracer but without cross-linker (DSS); lane 3, serum treated with tracer and cross-linker; lanes 4 and 5 show autoradiographic patterns generated by lanes 2 and 3; lane 6, autoradiography of isolated binding protein treated with tracer and cross-linker in the presence of 10^{-3} M of unlabeled [D-Lys^6, Pro^9-NEt]-sGnRH; lane 7, of isolated binding protein treated with tracer and cross-linker in the absence of unlabeled hormone.

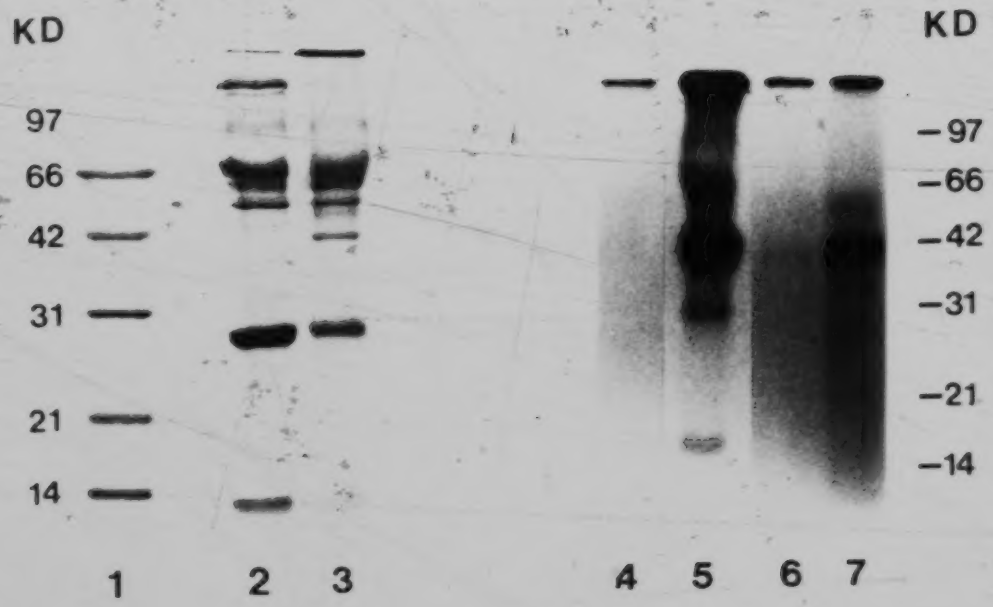

KD
97
66
42
31
21
14
1
2
3
4
5
6
7
KD
97
66
42
31
21
14

Fig. 3.5. Purification of the GnRH binding protein from fraction C (isolated with preparative PAGE) by HPLC. Affinity labeling of ^{125}I-sGnRH-L to GnRH-BP was carried out before injection into the HPLC column. HPLC column, Bio-Gel TSK-phenyl-5-PW (75 x 4.6 mm I. D.); conditions, linear gradient (0.5% B/min) where solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile; flow-rate, 1 ml/min; absorbance at 210 nm. The protein profile was shown by solid line and the radioactivity by the dashed line with solid circles.

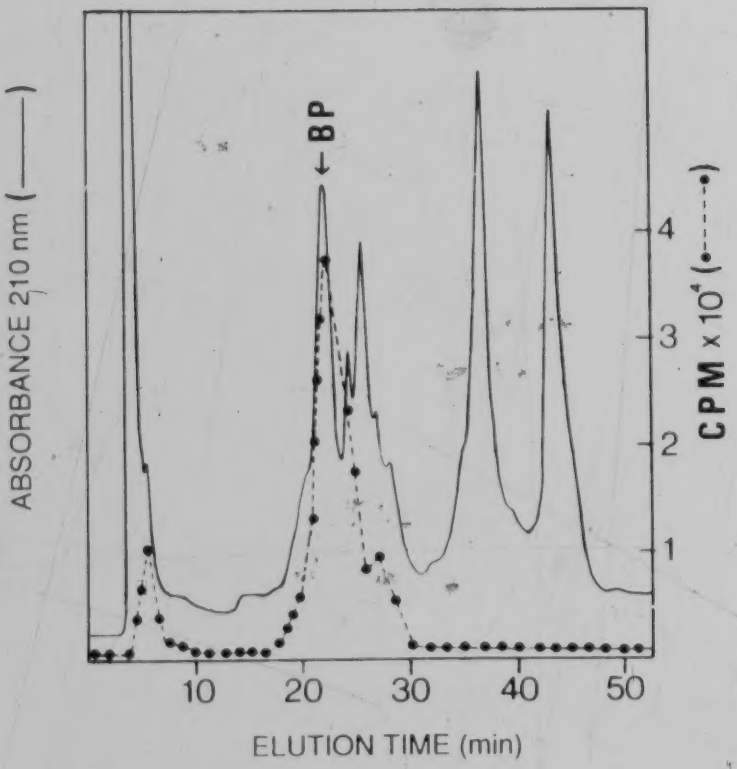

ABSORBANCE 210 nm (——)
← BP
CPM x 10⁴ (•-----•)
1
2
3
4
10
20
30
40
50
ELUTION TIME (min)

Fig. 3.6. Comparison of the GnRH-BP isolated by preparative gel with or without further purification by HPLC. After electrophoresis on a SDS gel (12% acrylamide) under reducing conditions, proteins contained in the gel were stained with coomassie blue (lanes 1-3) or with silver stain (lanes 4 and 5). Lane 1, Bio-Rad low molecular weight standards; lanes 2 and 4, the binding protein after HPLC purification; lanes 3 and 5, the binding protein isolated by preparative gel (fraction C) without further purification by HPLC.

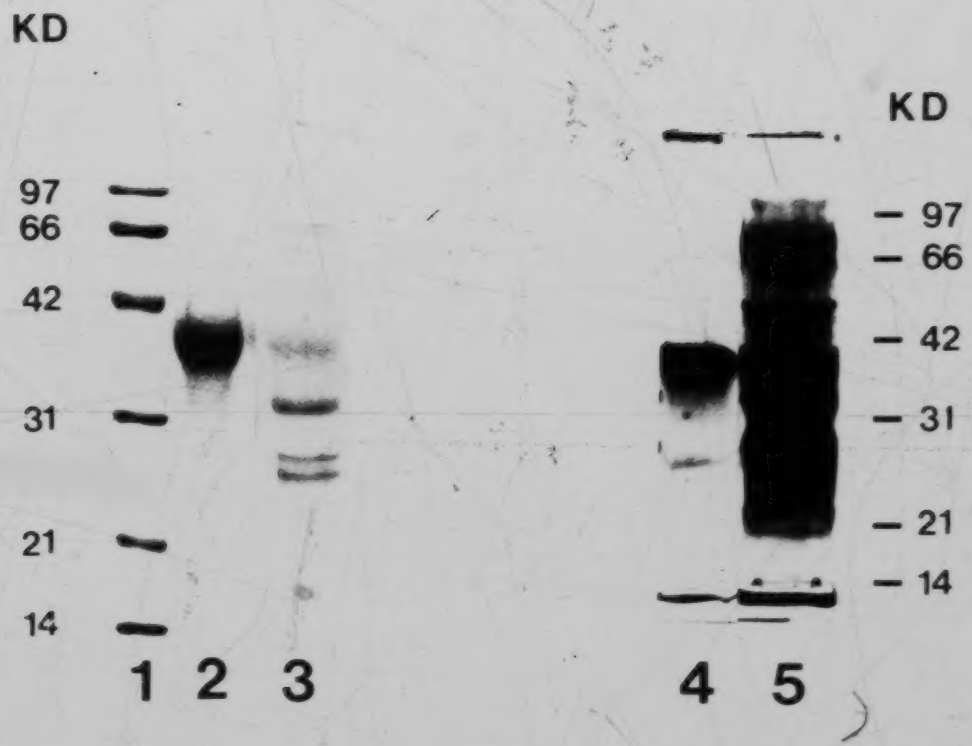

KD
97
66
42
31
21
14
1 2 3
KD
97
66
42
31
21
14
4 5

Fig. 3.7. Specificity of the isolated GnRH binding protein (fraction C). Displacement of tracer, ^{125}I-sGnRH-A, from the binding protein by the five known forms of GnRH, and sGnRH-A and sGnRH-L at concentration of 10^{-5} M. Values are mean ±SEM (n = 3). *, significantly different from 0 GnRH control, $p < 0.05$, Mann-Whitney U test, **, $p < 0.01$, ***, $p < 0.001$.

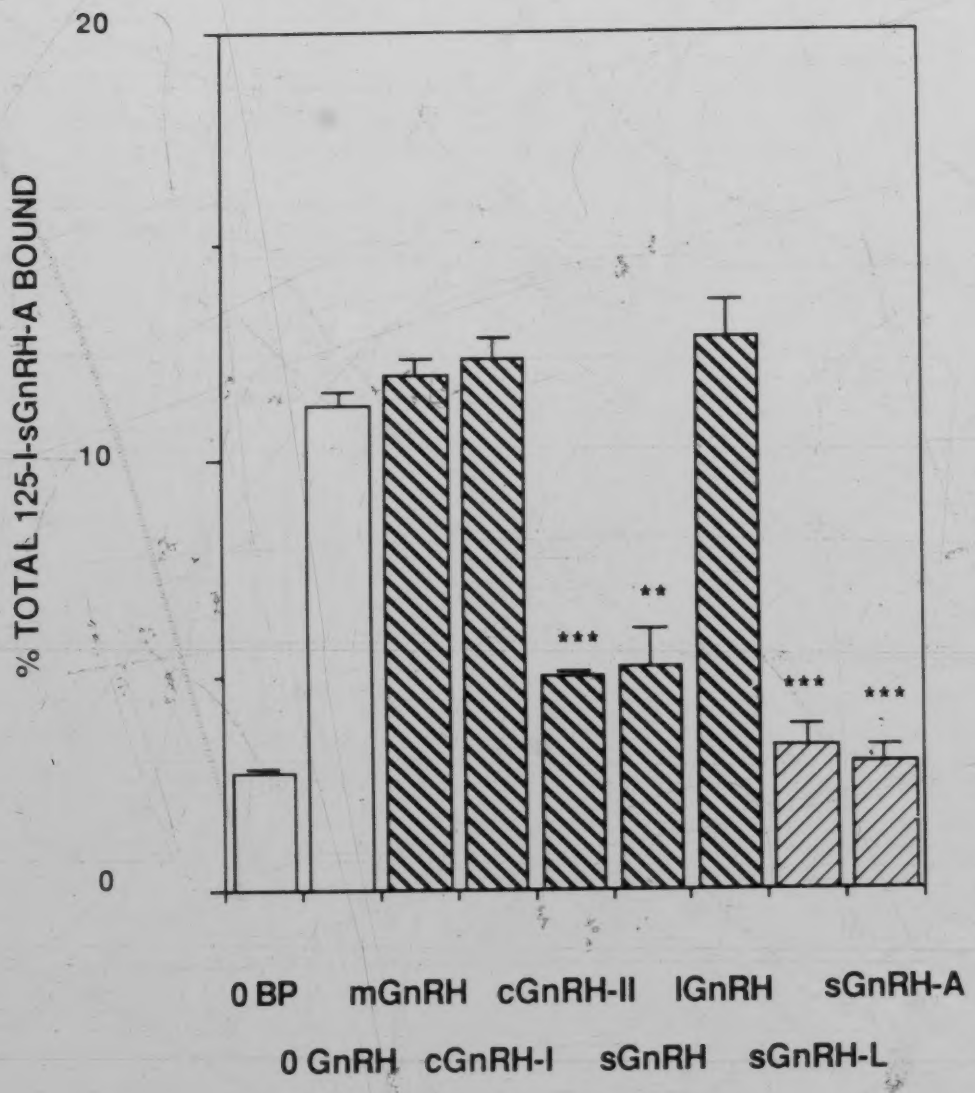
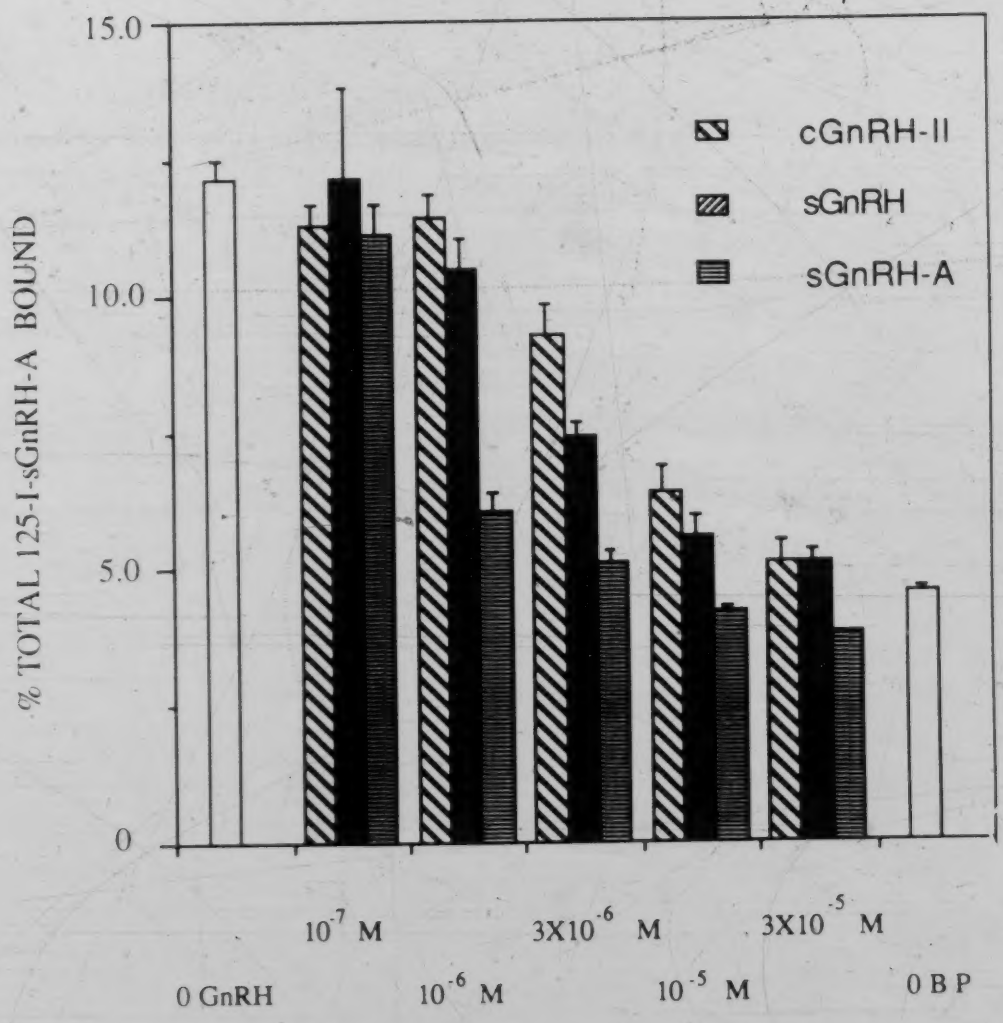

20
10
0
% TOTAL 125-I-sGnRH-A BOUND
***
**
***
***
0 BP
mGnRH
cGnRH-II
lGnRH
sGnRH-A
0 GnRH
cGnRH-I
sGnRH
sGnRH-L

Fig. 3.8. Dose-dependent displacement of ^{125}I-sGnRH-A from the isolated GnRH binding protein by cGnRH-II, sGnRH and sGnRH-A. The calculated ED_{50} values are 4.95×10^{-6} M, 2.19×10^{-6} M, 4.21×10^{-7} M for cGnRH-II, sGnRH and sGnRH-A, respectively. Values are mean±SEM (n = 3).


15.0
10.0
5.0
0
% TOTAL 125-I-sGnRH-A BOUND
cGnRH-II
sGnRH
sGnRH-A
0 GnRH
10^-7 M
10^-6 M
3X10^-6 M
10^-5 M
3X10^-5 M
0 B P

3.5 LITERATURE CITED

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4 CIRCULATING FORMS OF GONADOTROPIN-RELEASING HORMONE IN GOLDFISH SERUM

4.1 INTRODUCTION

The existence of multiple molecular forms of gonadotropin-releasing hormone (GnRH) in the brain of a single species has been well documented in a wide range of vertebrates, including birds, reptiles, amphibians, teleosts, chondrostean, elasmobranchs, and lamprey (King and Millar, 1987; Millar and King, 1987; Sherwood, 1986, 1987); in mammals the presence of multiple GnRH forms in the brain (King *et al.*, 1988; Stopa *et al.*, 1988) and pituitary neural lobe (Anthony *et al.*, 1987) have been reported, but the best evidence for a second form of mammalian GnRH is from the recent study on marsupial (King *et al*, 1989).

In teleosts, multiple forms of GnRH have consistently been found in the brain tissue of a single species (Gentile *et al.*, 1986; Peter *et al.*, 1990; Powell *et al.*, 1986; Yu *et al.*, 1988). Salmon GnRH ([Trp^7, Leu^8]-GnRH, sGnRH) is present in a wide range of teleost species, (Breton *et al.*, 1987; King and Millar,1985; Powell *et al.*, 1986; Sherwood, 1986; Sherwood and Lovejoy, 1989; Sherwood *et al.*, 1983, 1984), including goldfish (Yu *et al.*, 1988), although it is absent in African catfish (Sherwood and Lovejoy, 1989; Sherwood *et al.* 1989). In addition to sGnRH, chicken GnRH-II ([His^5, Trp^7, Tyr^8]-GnRH, cGnRH-II) has also been detected in brain extracts of a number of teleost species (Sherwood *et al.*, 1984), including goldfish (Yu *et al.*, 1988), by high pressure liquid chromatography (HPLC) and radioimmunoassay (RIA). A differential distribution of sGnRH and cGnRH-II has been found in the goldfish brain, using the combination of HPLC and RIA, with about equal amounts of the two peptides in forebrain regions and the pituitary, but a predominance of cGnRH-II in mid- and hind-brain regions (Yu *et al.*, 1988). A specific GnRH binding protein has been demonstrated in goldfish serum

(Chapters 2 and 3; Huang and Peter, 1988). Although King and Millar (1980) detected immunoreactive GnRH in dogfish blood, the presence of GnRH in the circulation of teleosts has not been investigated. The present study demonstrates the existence of sGnRH and cGnRH-II in goldfish serum using reverse phase HPLC and RIAs with antisera of different specificity.

4.2 MATERIALS AND METHODS

Serum samples

Goldfish, *Carassius auratus*, common and comet varieties, were purchased from Grassyforks Fisheries Co. (Martinsville, IN). The fish were held in 96-liter flow-through aquaria at 17±1 °C on a simulated natural (Edmonton) photoperiod for at least 4 weeks, and fed a commercial diet (EWOS).

Blood was collected in February from a combination of maturing males and females undergoing gonadal recrudescence. Fish were anesthetized in 0.05% tricaine methanesulfonate, and blood was collected by puncture of the caudal vasculature with a 25-gauge needle attached to a 1-ml syringe. Bacitracin was added to the blood samples to a final concentration of 2×10^{-5} M to inhibit peptidase activity (McKelvy et al., 1976). Serum was collected following centrifugation at 13,000 g for 5 min. Approximately 80 ml of serum was pooled and used immediately for extraction.

Extraction

The fresh serum was mixed with 2 M acetic acid (10:1 v/v), and centrifuged at 3,000g for 20 min to remove precipitated proteins. Supernatant collected was partially purified by loading onto a series of three C_{18} 'Sep-Pak' cartridges (Water's Associates, Milford, MA), previously activated using HPLC grade methanol. Each cartridge was washed with 15 ml of 0.25 M formic acid adjusted with triethylamine to pH 6.5 (TEAF). Material in each Sep-Pak cartridge was eluted with 30 ml 50% acetonitrile (CH_3CN) in TEAF. The eluates were defatted with petroleum ether (b. p. 30-60 °C). The volume of the final aqueous phase was reduced to 800 µl using a Speed Vac vacuum concentrator.

High Pressure Liquid Chromatography

Chromatography of goldfish serum extracts was performed on a Gilson liquid chromatograph system. An aliquot of 200 µl of serum extract was injected via a 200-µl injection loop into a Supelco C_{18} column (0.46 x 25 cm) with a C_{18} guard column. The mobile phase was acetonitrile (CH_3CN) in TEAF, starting with an isocratic initial elution of 17% CH_3CN for 10 min, increasing during the following 7 min to 24% CH_3CN, and staying at 24% CH_3CN for 43 min; the flow rate was 1 ml per min. The column outflow was collected in 1 ml fractions and aliquots assayed for immunoreactive GnRH (irGnRH) using two antisera (S-30-3 and 8NW4). Each injection of serum extract was preceded by a blank run in which TEAF (200 µl) was injected and 1 ml fractions collected under the same conditions as for the serum extract. Fractions from the blank run were assayed for irGnRH to determine whether there was any carry-over of GnRH from one run to another. Fresh standards were chromatographed between sample runs for comparison with the serum extract. Five hundred ng of synthetic mammalian (mGnRH, purchased from Sigma), sGnRH, [Gln8]-GnRH (cGnRH-I; Pennisula Laboratories, San Carlos, CA), cGnRH-II, lamprey GnRH ([Tyr^3, Leu^5, Glu^6, Trp^7, Lys^8]-GnRH, (lGnRH, a gift from R. Millar, University of Cape Town, South Africa) were mixed, diluted with TEAF to a final volume of 200 µl, and injected into the HPLC column under the same conditions used for serum extract.

Radioimmunoassay

Aliquots of 200 µl from each HPLC fraction were dried in a Speed Vac vacuum concentrator and assayed for immunoreactive GnRH (irGnRH). These samples were

reconstituted in 200 µl of assay buffer containing 10 mM Tris-HCl, 1 mM dithiothretol, and 0.5% bovine serum albumin (BSA), pH 7.4. The rabbit anti-cGnRH-II antiserum (8NW4, raised against synthetic cGnRH-II conjugated to bovine thyroglobulin in a rabbit) and anti-sGnRH antiserum (S-30-3, raised against synthetic sGnRH conjugated to bovine thyroglobulin in a rabbit) were used at final concentrations of 1:100,000 and 1:250,000, respectively.

Chicken GnRH-II was iodinated using the Chloramine T method. One mCi $Na^{125}I$ in 50 µl of 0.16 M phosphate buffer (pH 7.4) was added to a conical vial containing 5 µg cGnRH-II in 10 µl of 0.1 N acetic acid. The reaction was initiated by the addition of 5 µg Chloramine T (Sigma Chemical Co. St. Louis, MO) in 10 µl of 0.16 M phosphate buffer, pH 7.4. Following 2 min of gentle agitation, the reaction was terminated by dilution with the addition of 200 µl starting elution buffer (2 mM ammonium acetate buffer, pH 4.5). A carboxymethyl cellulose (fine mesh, Sigma) cation exchange column (0.6 x 6 cm) was used to purify the ^{125}I-cGnRH-II; the column was eluted with 15 ml of 2 mM ammonium acetate buffer (pH 4.5) followed by 60 ml of 200 mM ammonium acetate buffer (pH 4.5) at a flow rate of 0.75 ml per min, and 3-ml fractions were collected. The elution profiles of radioactivity and immunoactivity are shown in Figure 4.1-A. Specific activity of the ^{125}I-cGnRH-II was carried out on a routine basis by a self-displacement radioimmunoassay; the mass-radioactivity relationship was estimated at 1200 µCi/µg.

In the cGnRH-II RIA, 100 µl of anti-cGnRH-II antiserum (8NW4, initial dilution 1:20,000) containing 5% normal rabbit serum in assay buffer was added to each incubation tube containing 200 µl of reconstituted sample or 200 µl of standard, followed by the addition of 200 µl of ^{125}I-cGnRH-II (about 15,000 cpm/200 µl) in assay buffer, and then incubated for 48 hours at 4 °C with periodic agitation. All samples were assayed in duplicate. Following this initial incubation, 200 µl of goat anti-rabbit gamma globulin

antiserum (Calbiochem) diluted 1:20 in assay buffer was added to each assay tube. After overnight incubation at 4 °C, the incubation tubes were centrifuged for 25 min at 1000g ,the supernatant decanted, and the radioactivity in the pellet fraction counted. The minimum sensitivity of the RIA, defined as the minimum concentration of cGnRH-II resulting in significant ($p<0.01$) displacement of the specifically bound ^{125}I-cGnRH-II (Reuter *et al.*, 1978) was calculated as 2 pg/tube. The concentration of cGnRH-II resulting in 50% displacement (ED_{50}) of the specifically bound ^{125}I-cGnRH-II was calculated as 11.2±1.1 pg/tube (mean ± SEM, n=3). The within assay variability for brain tissue samples containing 34.6 or 83.3 pg ir-cGnRH-II per ml was calculated as percent coefficient of variation (% CV) of 5.4 and 8.9 (n=4), respectively; the between assay variability for each sample was calculated as % CV of 7.2 and 15.7 (n=3), respectively.

Iodination and purification of labelled sGnRH followed the procedure described for cGnRH-II, except that 100 mM ammonium acetate buffer was used as the second elution buffer. The profiles of radioactivity and immunoactivity are shown in Figure 4.1-B. The specific activity of ^{125}I-sGnRH was estimated as 1,100 μCi/μg by a self-displacement radioimmunoassay. Anti-sGnRH antiserum (S-30-3) raised against synthetic sGnRH was used at a final dilution of 1:250,000 in the RIA. The procedure for sGnRH RIA follows that described for cGnRH-II RIA. The minimum sensitivity of the RIA was calculated as 2 pg/tube, and the ED_{50} was calculated as 20.8±1.8 pg/tube (mean ± SEM, n=3). The within assay variability for brain tissue samples containing 75 or 200 pg ir-sGnRH per ml was 4.9 and 6.9 (% CV, n=4), respectively; the between assay variability for each sample was 8.1 and 12.6 (% CV, n=4), respectively. Linear displacement curves produced by synthetic GnRH peptides and the HPLC fractions were constructed using logit-log transformation. Parallelism between the various displacement curves was determined by using analysis of covariance or by comparing the slopes of the two regression lines using Student *t*-test (Snedcor and Cochran, 1976). Table 4.1 summarizes the crossreactivity of the various GnRH peptides with antisera 8NW4 and S-30-3.

4.3 RESULTS

Reverse phase HPLC of goldfish serum extract followed by RIA of each fraction with antisera 8NW4 and S-30-3 revealed two peaks of GnRH immunoactivity (Figure 4.2). The two immunoreactive peaks coeluted with synthetic cGnRH-II and sGnRH, respectively. Chicken GnRH-I and mGnRH, both of which cross-react substantially with antiserum S-30-3 (see Table 4.1) were not detectable in the HPLC fractions; lGnRH, which crossreacted with S-30-3 to a small extent, was also not detectable in the HPLC fractions.

To further identify the immunoactivity of ir-GnRH materials in the two peaks, serial dilutions of the early eluting peak were assayed using the cGnRH-II RIA with antisera 8NW4; the late eluting peak was assayed by sGnRH RIA with antiserum S-30-3. The GnRH immunoactivity in the early eluting peak resulted in a displacement curve parallel to that of synthetic cGnRH-II (Figure 4.3, upper panel). The GnRH immunoactivity in the late eluting peak resulted in a displacement curve parallel to that of synthetic sGnRH (Figure 4.3, lower panel). The HPLC separation and RIA data together indicate that the first HPLC peak is chromatographically and immunologically similar to cGnRH-II and the second peak to sGnRH, respectively. From the extract equivalent to 3 ml of serum, the amount of cGnRH-II measured in the first peak using antiserum 8NW4 is 54 pg, indicating a serum concentration of 18 pg/ml; the amount of sGnRH measured in the late eluting peak using antiserum S-30-3 is 17.7 pg, indicating a serum concentration of 5.9 pg/ml, without correction for extraction efficiency (table 4.2).

4.4 DISCUSSION

The demonstration in the present study of cGnRH-II and sGnRH in serum from goldfish is in agreement with previous studies showing that these two forms are present in extracts of the goldfish brain, spinal cord, and pituitary (Yu *et al.*, 1988). Although antiserum S-30-3 used in the sGnRH RIA crossreacts substantially with mGnRH and cGnRH-I (Table 4.1), no detectable mGnRH or cGnRH-I was found in goldfish serum; mGnRH, cGnRH-I and lGnRH have not been reported in the brain extracts of goldfish (Yu *et al.*, 1988).

This is the first report of two forms of GnRH in the circulation of a vertebrate. In chicken, both cGnRH-I and cGnRH-II are present in brain; however, in contrast to cGnRH-I, cGnRH-II is not detected in the median eminence and is not released into the portal system (Mikami *et al.*, 1988; Sharp *et al.*, 1988). On this basis cGnRH-I was suggested to have a neuroendocrine function and cGnRH-II to have a neurotransmitter or neuromodulator function in chicken. In goldfish, a functional hypothalamo-hypophysial blood portal system is lacking and the pituitary is directly innervated by immunoreactive GnRH fibers (Kah *et al.*, 1984, 1986). The presence of a substantial amount of both cGnRH-II and sGnRH in the pituitary of goldfish (Yu *et al.*, 1988) suggests a role for both GnRH forms in the regulation of pituitary functions. It has been demonstrated that sGnRH, mGnRH, and analogs of each stimulate growth hormone (GH) as well as gonadotropin (GtH) release *in vivo* as well as *in vitro* from perifused fragments of the goldfish pituitary (Marchant and Peter, 1989; Marchant *et al.*, 1989). More recently, it has been shown that cGnRH-II is more potent than sGnRH in releasing GtH, but equipotent in releasing GH, from cultured dispersed cells of the goldfish pituitary (Chang *et al.*, 1990; Peter *et al.*, 1990). The ED_{50} dosage for sGnRH and cGnRH-II in releasing GtH and GH in the *in vitro* test systems used is in the nanomolar range, with the lower ends of the dose response curves in the mid-picomolar range. Notably, the concentrations of sGnRH and cGnRH-II detected in goldfish serum in the present study coincides with the minimal dosages found effective in stimulating GH and GtH release *in vitro*. Although we do not know at this time whether circulating sGnRH and cGnRH-II have a neuroendocrine function in goldfish, the present study has provided a starting point for further evaluation

of the possible physiological function of circulating GnRH, particularly in regard to its roles as a hormone.

The presence of a specific GnRH binding protein has been shown in our previous studies (Huang and Peter, 1988; Chapers 2 and 3). The binding protein recognizes both sGnRH and cGnRH-II, but not other forms of naturally occurring GnRH. It is of high capacity, and it is unsaturated by the low concentrations of circulating GnRH in goldfish. The binding of GnRH to its carrier protein may prolong the half-life of GnRH in circulation in goldfish (Chapter 6), and extraction steps are taken to separate the circulating GnRH from the binding protein in this study.

The origin of the circulating GnRH is not known. Notably the serum concentrations of cGnRH-II are higher than that of sGnRH, in contrast to the pituitary where the concentrations of cGnRH-II are lower than that of sGnRH in male goldfish and in females where similar concentrations of both forms are found (Yu *et al.*, 1988). This suggests that the two forms of GnRH may be differentially released into the circulation. Whether the circulating GnRH represents "spillover" from the pituitary is not known; however, the mismatch between pituitary content and serum concentrations of the two forms of GnRH would suggest other origins may also contribute to the circulating pools of cGnRH-II and sGnRH.

TABLE 4.1

CROSSREACTIVITY OF ANTISERA TO VARIOUS VERTEBRATE GNRH PEPTIDES IN RADIOIMMUNOASSAYS

Antiserum	Iodinated Tracer	GnRH Standard	Percent crossreactivity (%)				
			sGnRH	mGnRH	cGnRH-I	cGnRH-II	lamprey GnRH
8NW4	cGnRH-II	cGnRH-II	5.0	0	0	100	0.5
S-30-3	sGnRH	sGnRH	100	69.7	82.1	9.3	1.0

*Percentage crossreactivity was measured at B/B0 =50%

TABLE 4.2

COMPARISON OF THE EARLY ELUTING cGnRH-II-LI AND THE LATE ELUTING sGnRH-LI IN EXTRACTS OF GOLDFISH SERUM (3 ml) USING TWO DIFFERENT RIA

Antiserum	Iodinated tracer	Early Eluting cGnRH-LI (pg)	Late Eluting sGnRH-LI (pg)
8NW4	cGnRH-II	54.0	n. d.
S-30-3	sGnRH	47.1	17.7

n. d.: Not detectable.
Values are not corrected for extraction efficiency

Fig. 4.1. Profiles of radioactivity following chromatography of the iodination reaction mixture on CM cellulose ion exchange column (triangles). The percentage of radioactivity in various fractions specifically bound by antisera (upper panel : 8NW4 in 1 : 1,500 final dilution; lower panel : S-30-3 in 1 : 10,000 final dilution) is also indicated (rectangles). The switch of elution buffer from 2 mM to 200 mM (upper panel) and 2 mM to 100 mM (lower panel) ammonium acetate is indicated by the arrows.

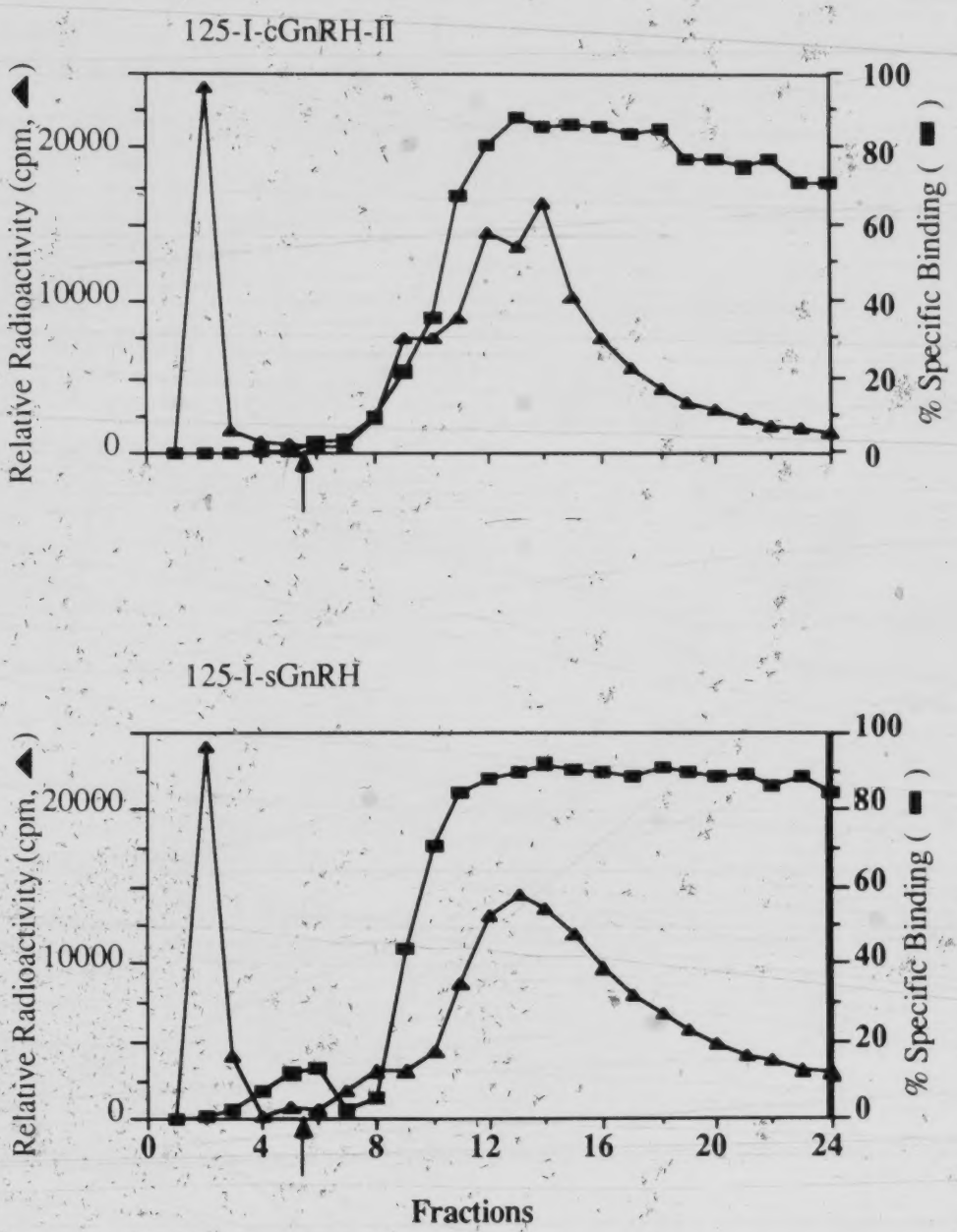

125-I-cGnRH-II
Relative Radioactivity (cpm, ▲)
% Specific Binding (■)
0
10000
20000
0
20
40
60
80
100
125-I-sGnRH
Relative Radioactivity (cpm, ▲)
% Specific Binding (■)
0
10000
20000
0
20
40
60
80
100
0
4
8
12
16
20
24
Fractions

Fig. 4.2. Reverse phase HPLC of extracts of goldfish serum (original volume = 20 ml) and RIA of GnRH fractions. Elution positions of mGnRH and lGnRH (1), cGnRH-I (2), cGnRH-II (3), and sGnRH (4) are indicated. Each column represents the total amount of ir-GnRH in each 1-ml HPLC fraction. The mobile phase was acetonitrile in TEAF, pH 6.5. Percent acetonitrile is shown as a broken line. Aliquots (200 µl) of the HPLC fractions were assayed with antiserum 8NW4 (upper panel) in a cGnRH-II RIA, and with antiserum S-30-3 (lower panel) in a sGnRH RIA.

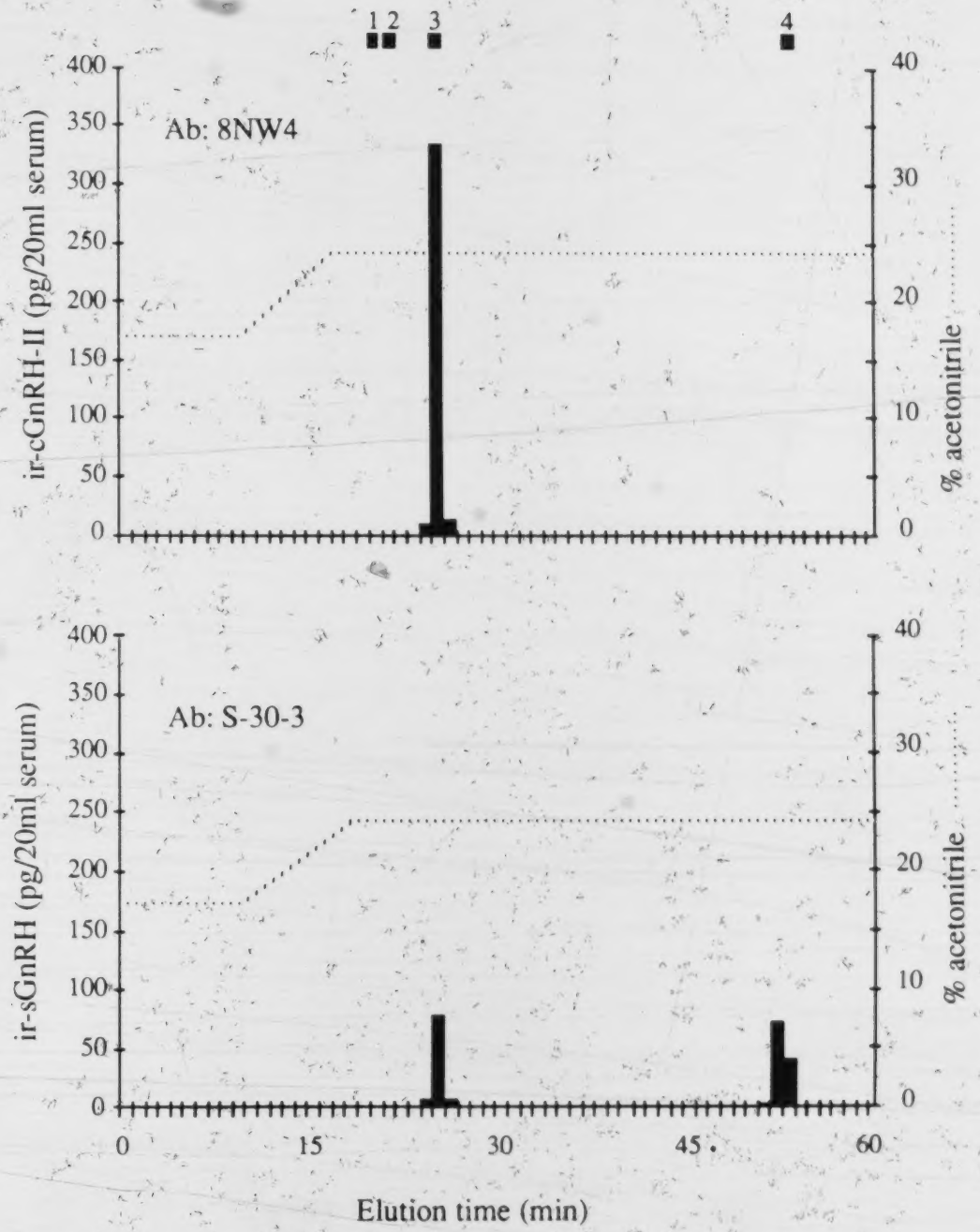
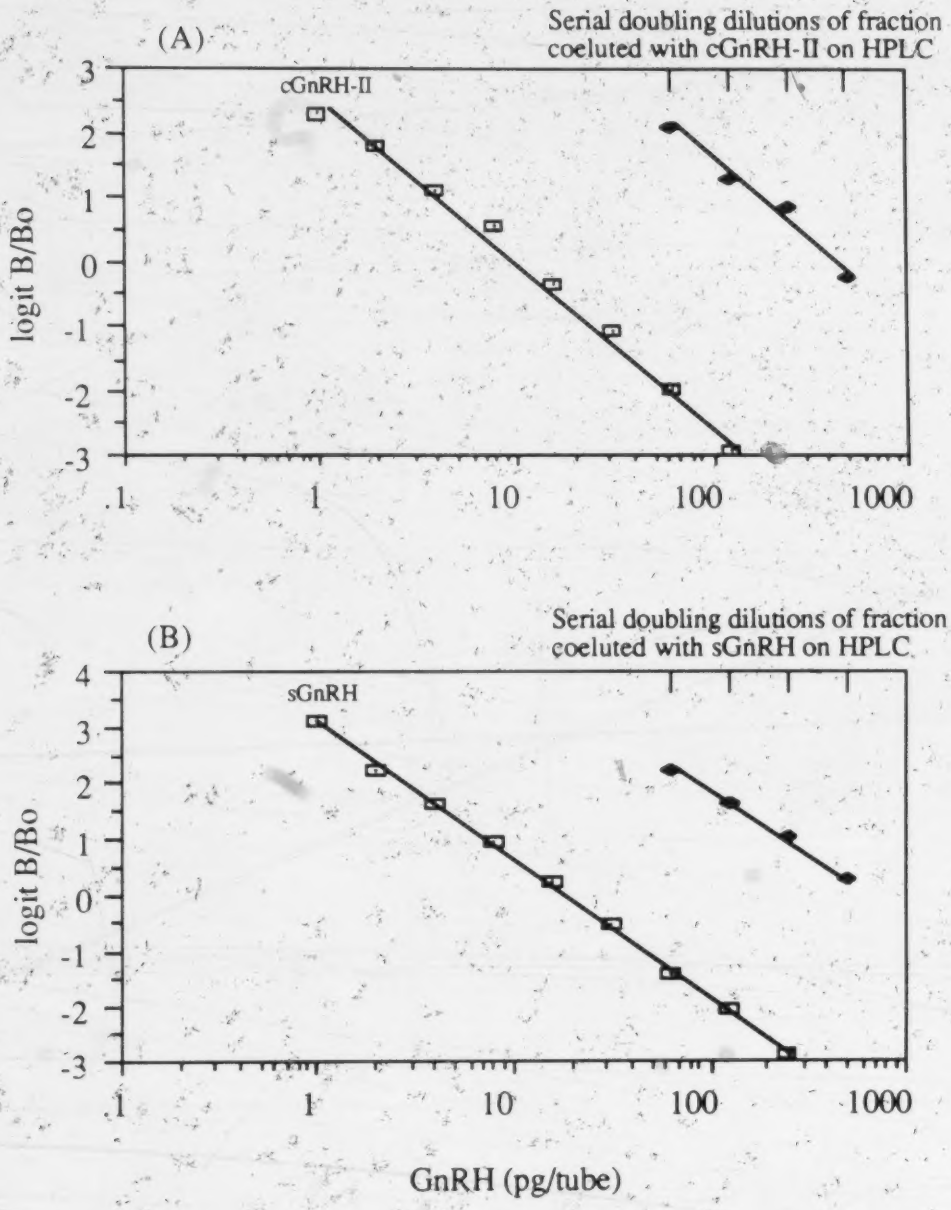

1 2 3
4
Ab: 8NW4
ir-cGnRH-II (pg/20ml serum)
% acetonitrile
Ab: S-30-3
ir-sGnRH (pg/20ml serum)
% acetonitrile
0
50
100
150
200
250
300
350
400
10
20
30
40
0
15
30
45
60
Elution time (min)

Fig. 4.3. Radioimmunoassay of HPLC fractions from goldfish serum.

Upper panel: Competitive displacement of ^{125}I-cGnRH-II from antiserum 8NW4 by synthetic cGnRH-II and the "early" eluting immunoreactive peak in extracts of goldfish serum. Each point represents the mean of duplicate determinations.

Lower panel: Competitive displacement of ^{125}I-sGnRH from the antiserum S-30-3 by synthetic sGnRH and the "late" eluting immunoreactive peak in extracts of goldfish serum. Each point represents the mean of duplicate determinations.


(A)
Serial doubling dilutions of fraction coeluted with cGnRH-II on HPLC
cGnRH-II
logit B/Bo
3
2
1
0
-1
-2
-3
.1
1
10
100
1000
(B)
Serial doubling dilutions of fraction coeluted with sGnRH on HPLC
sGnRH
logit B/Bo
4
3
2
1
0
-1
-2
-3
.1
1
10
100
1000
GnRH (pg/tube)

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5 SEASONAL CHANGES AND EFFECTS OF HORMONE TREATMENT ON CONTENT OF THE GNRH BINDING PROTEIN IN GOLDFISH SERUM

5.1 INTRODUCTION

In previous research I demonstrated the presence and characteristics of a binding protein specific for gonadotropin-releasing hormone (GnRH-BP) in goldfish serum (Chapter 2). This GnRH-BP was isolated by native polyacrylamide gel electrophoresis, and further purified by high pressure liquid chromatography (Chapter 3). All of this previous research was based on large pools of serum samples from mixed sex animals. This did not allow quantification of differences in the amount of GnRH-BP between individuals. In this chapter, the differences in serum titer of GnRH-BP between male and female animals, and between animals at different stages of gonadal development were investigated. The effects of estradiol (E_2) implant or long term [D-Arg^6, Pro^9-NEt]-salmon GnRH (sGnRH-A) treatment on the serum titer of GnRH-BP were also investigated.

5.2 MATERIALS AND METHODS

Animals

Goldfish of the common or comet varieties were purchased from Grassyforks Fisheries (Martinsville, IN), or Ozark Fisheries (Stoutland, MO) Fish were maintained in 96-liter flow-through aquaria and acclimated to the designed experimental conditions for at least four weeks prior to treatment. Fish were fed Ewos trout pellets and Nutrafin flaked food *ad libitum* at random times during the light period.

Experiment 1.

In this experiment differences in serum titer of GnRH-BP between male and female goldfish were examined. Blood samples were taken early in May from sexually mature (completed gonadal recrudescence) male and female goldfish maintained at 17±1 °C under a simulated natural (Edmonton) photoperiod for a minimum of four weeks. Fish were anethetized in 0.05% tricaine methanesulfonate, and blood was taken by puncture of the caudal vasculature with a 25-guage needle attached to a 1-ml syringe. The blood was allowed to clot at 4 °C for several hours, and serum collected following centrifugation at 13,000g for 5 min. Individual serum samples were stored at -20 °C until use. Following blood sampling, fish were sacrificed by spinal transection and the gonads removed for gonadosomatic index (GSI) determination.

For measurement of the serum titer of GnRH-BP, the ligand binding assay was carried out as described previously (Chapters 2 and 3). Briefly, individual serum samples diluted 1 : 120 in 10 mM Tris-HCl buffer, containing 1 mM dithiothreitol, 0.5% BSA , pH7.6, were incubated with approximately 30,000 cpm of ^{125}I-sGnRH-A at 4 °C for 10 hours in a final volume of 600 µl. At the end of the incubation, a 500 µl aliquot of incubate was used for determination of the total binding level. Labeled ligand bound to serum GnRH-BP was

separated from the free ligand using a mini-column of Sephadex G-50. The bound fraction was eluted from the mini-column in the first peak and collected to be counted in a gamma scintillation counter (Model MS 4/600, Micromedic System Ico. Ltd, Horsham, PA). Protein content measurement was not carried out] for this experiment.

Experiment 2.

In this experiment the effects of long term treatment of sGnRH-A on the serum titer of GnRH-BP were tested. Mixed sex fish in early stages of gonadal recrudescence (December) were acclimated to a 16 hours light : 8 hours dark photoperiod at 20±1 °C for a minimum of four weeks. Pretreatment blood samples were taken shortly before the first injection. Serum samples were collected as described in Experiment 1. The sGnRH-A was dissolved in acidified (pH 6.0) fish physiological solution (PS; Burnstock, 1958) for intraperitoneal injection at a dosage of 0.1 µg/g body weight. Injection volumes of PS in control fish, and of sGnRH-A dissolved in PS, were 5µl/g body weight. In this experiment each fish received a total of 7 injections at 7 day intervals each. At 6 hours after each injection, blood samples were taken for measurement of serum GtH levels by double antibody radioimmunoassay (Peter *et al.*, 1984) Two days after the last injection, serum samples were collected for comparison of the serum titer of GnRH-BP with the pretreatment samples in a ligand binding assay. No measurement of serum protein content was carried out for this experiment.

Experiment 3.

In this experiment the seasonal variations of serum protein content and serum titer of GnRH-BP in male and female goldfish were examined. Groups of male and female goldfish were acclimated to a simulated natural (Edmonton) photoperiod at 17±1 °C for a minimum of four weeks. Blood samples were taken at three separate times of the year:

early February (gonads recrudescing), mid April (gonads completed recrudescence), and mid June (sexually regressed). Individual serum samples were collected and the ligand binding assay carried out as described in Experiment 1. Protein content of serum samples was determined by Bradford's method, using bovine serum albumin (BSA) as standards (Bradford, 1976).

Experiment 4.

The effects of estradiol implantation on serum protein content and serum titer of GnRH-BP were investigated in this experiment. Sexually regressed female fish were kept in 96-liter flow-through aquaria and maintained on a photoperiod of 16 hours light : 8 hours dark at a water temperature of 20±1 °C for a minimum of four weeks. Regressed female fish (September) were given an intraperitoneal implant of either a solid blank silastic pellet or a solid silastic pellet containing 100 μg/g body weight E_2 (Pankhurst *et al.*, 1986).

Sample measurement and data analysis

The serum titer of GnRH-BP was quantified by the ligand binding assay. In Experiments 1 and 2, differences in the percentage of specific binding of labeled ligand to the GnRH-BP in serum (1 : 120 dilution) between males and females (Experiment 1), or between control animals and hormone treated animals (Experiment 2) were analyzed by Mann-Whitney U test. Differences in serum GtH levels between PS and sGnRH-A groups at each sample time are tested by Student's *t* test.

In Experiments 3 and 4, differences in serum protein content were analyzed by analysis of variance and Duncan's multiple range test, or by Student's *t* test, as appropriate. Differences in specific binding between groups were tested by Mann-Whitney U test.

5.3 RESULTS

Experiment 1.

Labeled ligand (^{125}I-sGnRH-A) was bound by diluted (1 : 120) serum samples of both male and female goldfish. Although there was variation between individuals in terms of serum titer of GnRH-BP, every individual serum sample demonstrated specific binding ability (significantly increased binding compared to controls, in which goldfish serum was not added). The specific binding of tracer to serum samples, expressed as a percentage of total labeled sGnRH-A added, was not significantly different ($p>0.05$) between males and females (Fig. 5.1).

Experiment 2.

In sexually regressed fish (Experiment 2, Fig. 5.2), there were no significant differences ($p>0.05$) in serum titer of GnRH-BP between the groups at pretreatment (initial samples). Animals treated with sGnRH-A (0.1 µg/g body weight) had significantly higher ($p<0.05$) serum GtH levels at 6 hours post-injection compared to PS injected controls (Fig. 5.3). After a total of 7 injections at 7 day intervals each, the sGnRH-A treated animals had a similar serum titer of GnRH-BP compared to the control group (Fig. 5.2).

Experiment 3.

In male fish, the serum protein content of sexually mature fish was significantly higher ($P<0.01$) than that of sexually regressed or recrudescing fish (Fig. 5.4). There were no significant differences in serum titer of GnRH-BP between the groups (Fig. 5.5). Every individual serum sample showed measurable specific binding at 1 : 120 dilution, indicating that the GnRH-BP was present in every individual animal at all stages of gonadal development. When the serum titer of GnRH-BP was standardized to a 30 mg/ml protein

content, mature males had a lower but not significantly different serum titer of GnRH-BP (data not shown).

In females, sexually mature fish had a higher ($P<0.05$) serum protein content than regressed fish. (Fig. 5.6). There were no significant differences in serum titer of GnRH-BP between the groups (Fig. 5.7).

Experiment 4.

Sexually regressed female goldfish implanted with E_2 (100 μg/g body weight) had a significantly higher serum protein content than the control group (blank implant) at the 7 day ($P<0.001$) and 35 day ($P<0.001$) sample times (Fig. 5.8). As described previously, individual serum samples were diluted (1 : 120, v/v) and tested in the ligand binding assay; there were no significant differences in the serum titer of GnRH-BP between E_2 and blank implanted fish at 7 or 35 days (Fig. 5.9). When the serum titer of GnRH-BP was standardized to a 30 mg/ml protein content, the abundance of GnRH-BP relative to total protein content was significantly lower ($p<0.05$) in E_2 treated fish compared to blank control fish (data not shown).

5.4 DISCUSSION

In the previous Chapters (Chapters 2-4), all experiments were carried out using pooled serum samples. Although no differences in the characteristics of the GnRH-BP were found from pool to pool, it was not possible to rule out changes in serum titer of GnRH-BP between individual fish. In the present chapter, individual serum samples were collected and used in the ligand binding assay for determination of the serum titer of GnRH-BP. The four experiments in this chapter demonstrated that a measurable amount of GnRH-BP is consistently detected in every individual goldfish serum sample, although there are variations in titer between individual samples.

Experiment 1 demonstrates that the GnRH-BP is present in serum from both male and female goldfish, and no significant differences in the serum titer of GnRH-BP were found between the sexes (Fig. 5.1). Similar observations were reported in human corticotropin-releasing factor binding protein (CRF-BP), which is a specific carrier protein for human CRF-41. The CRF-BP was first demonstrated in late gestational maternal plasma (Linton and Lowry, 1986), where it coexists with substantially elevated plasma levels of CRF-41 (Schulte and Healy, 1985; Campbell *et al.*, 1987). However, subsequent studies indicate that the binding protein is also present in non-saturable quantities in normal male plasma, where the endogenous CRF content is low (Linton *et al.*, 1988; Orth and Mount, 1987).

Experiment 2 demonstrates that long term treatment with sGnRH-A does not affect the serum titer of GnRH-BP (Fig. 5.2). This is similar to the finding by Tharandt *et al.* (1979) that there are no significant changes in the titer of nonspecific mammalian GnRH (mGnRH) binding in human serum after 2 months of treatment with a potent mGnRH analog. In both cases the most important result was the absence of any increase of binding of diluted sera, which might have pointed towards formation of an antibody to the analog. Results in Experiment 2 also indicate that the serum titer of GnRH-BP was not affected by

the secondary effect of high levels of GtH resulting from prolonged stimulation by sGnRH-A. This is in contrast to the sex steroid binding globulin in human, where androgens and estrogens both can change the concentration of the binding protein, which in turn influences the delivery of sex steroids to both target and metabolizing tissues (Belgorosky and Rivarola, 1982).

As indicated in Chapter 3, the GnRH-BP is not a major component of goldfish serum proteins (Figs. 3.1 and 3.2). Fig. 5.4 shows that sexually mature (completed gonadal recrudescence) male fish had a higher serum protein content than sexually recrudescing and regressed fish, and, in Fig. 5.6 , that sexually mature female fish had a higher serum protein content than in sexually regressed fish, reflecting changes in some major components of serum proteins. However, the serum titer of GnRH-BP was not different between sexually mature and regressed male or female fish (Figs. 5.5 and 5.7, respectively). This is in agreement with our previous findings that the GnRH binding in goldfish serum is not due to a component of any of the major serum proteins, and that the GnRH-BP accounts for only a small portion of the total protein content in goldfish serum (Figs 3.1 and 3.2). Unlike the changes in the total protein content, the serum titer of GnRH-BP did not change in different stages of reproductive cycle in both males and females, suggesting that the titer of GnRH-BP is not fine tuned to the changes related to gonadal development. It is unlikely that the GnRH-BP levels will change rapidly, since it is already present with high capacity in goldfish serum (Chapter 2), and is not saturated by the low endogenous levels of GnRH detected in the circulation (Chapter 4).

When sexually regressed female goldfish were implanted with E_2 (Experiment 4), the serum total protein content was doubled (Fig. 5.8). Since the vitellogenin (Vg) content in the serum samples was not measured, it is not known what proportion of the E_2 induced protein content (over 30 mg/ml) was contributed by Vg. However, it is well documented that Vg in fish is normally phospholipoglycoproteins, and their synthesis is induced in the

liver by estrogens in both male and female fish (Wallace and Selman, 1981; Wiegand, 1982; Ng and Idler, 1983). Moreover, Vg has been claimed to be the only serum protein that is induced by estrogen in Xenopus (Wallace, 1970). The majority of the increased protein content in serum in the E_2-treated fish in the present experiment is likely due to the increased secretion of vitellogenin (Vg) by liver, and the absence of Vg incorporation into the previtellogenic oocytes of the fish used in the present experiment may favor the accumulation of high levels in the serum. In the literature, the levels of Vg reported using RIA reached 100 mg/ml in sexually mature rainbow trout (Sumpter, 1985), and in the brown trout, *Salmo trutta*, Crim and Idler (1978) found levels ranging from 5 to 80 mg/ml during the reproductive cycle. Using quantitative immunoelectrophoresis, Goedmakers and Verboom (1974) found 1 to 48 mg/ml Vg during vitellogenesis in the female pike *Esox lucius*. The GSI in the E_2 treated animals was not different from the blank control animals 35 days after the implantation, suggesting the absence of Vg incorporation into the ovary in the E_2 treated animals, supporting the speculation that accumulated Vg may account for the majority of the E_2 induced serum protein. If Vg is assumed to be the major component induced by E_2 implant, then Vg is not associated with GnRH binding in goldfish serum because the serum titer of GnRH-BP did not increase while a large amount of Vg was accumulated in the serum.

Results in this chapter support the idea that the GnRH binding in goldfish serum is due to a specific protein, the titer of which does not change during reproductive cycles at the same time as there are major changes in the overall protein content of the serum. Indeed, the serum titer of GnRH-BP was not affected by high levels of E_2, sGnRH-A or sGnRH-A induced GtH. This GnRH-BP is present with high capacity in both males and females, coexisting with low concentrations of circulating GnRH(Peter, *et al.*, 1990; Chapter 4), and, consequently, unlikely to be closely regulated.

Fig. 5.1 Comparison of serum titer of GnRH-BP in male and female goldfish. Individual serum samples, collected from sexually mature male and female goldfish early in May, were diluted 1 : 120 and incubated with ^{125}I-sGnRH-A in the ligand binding assay. The specific binding of tracer to serum samples, expressed as a percentage of total labeled sGnRH-A added, is not significantly different between males and females ($p>0.05$, Mann-Whitney U test). Each sample was tested in triplicates. Number of fish in each group is indicated.

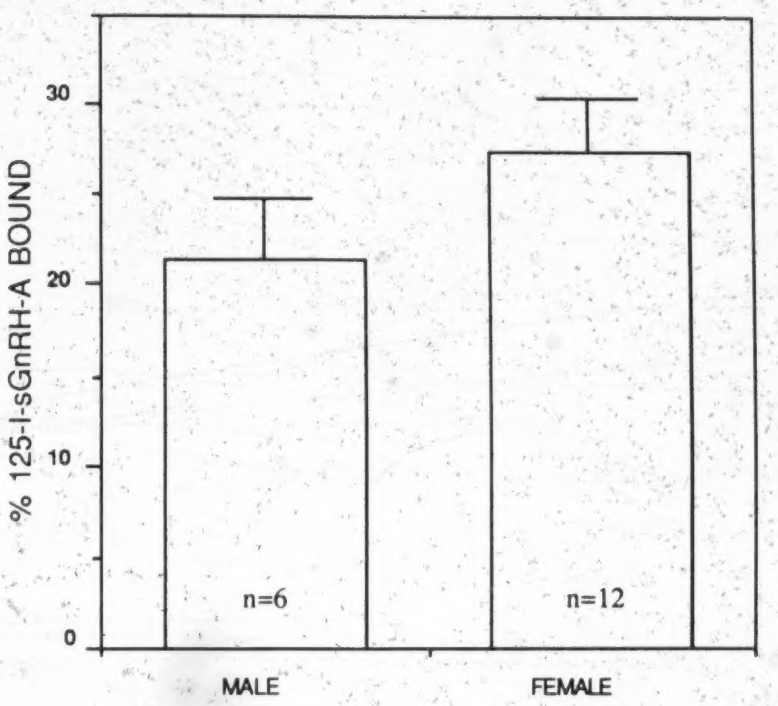

% 125-I-sGnRH-A BOUND
30
20
10
0
n=6
n=12
MALE
FEMALE

Fig. 5.2 Effects of long term treatment of sGnRH-A on the serum titer of GnRH-BP. Fish received a total of 7 ip injections of PS or sGnRH-A (0.1 μg/g body weight). Blood samples were taken before the first injection (Before treatment) and 2 days after the 7th injection 51 days later (After treatment). Serum samples were diluted 1 : 120 and tested in triplicate in the ligand binding assay. There were no significant differences between the two groups before treatment or after treatment ($p>0.05$, Mann-Whitney U test). Values are mean±SEM ($n = 10$).

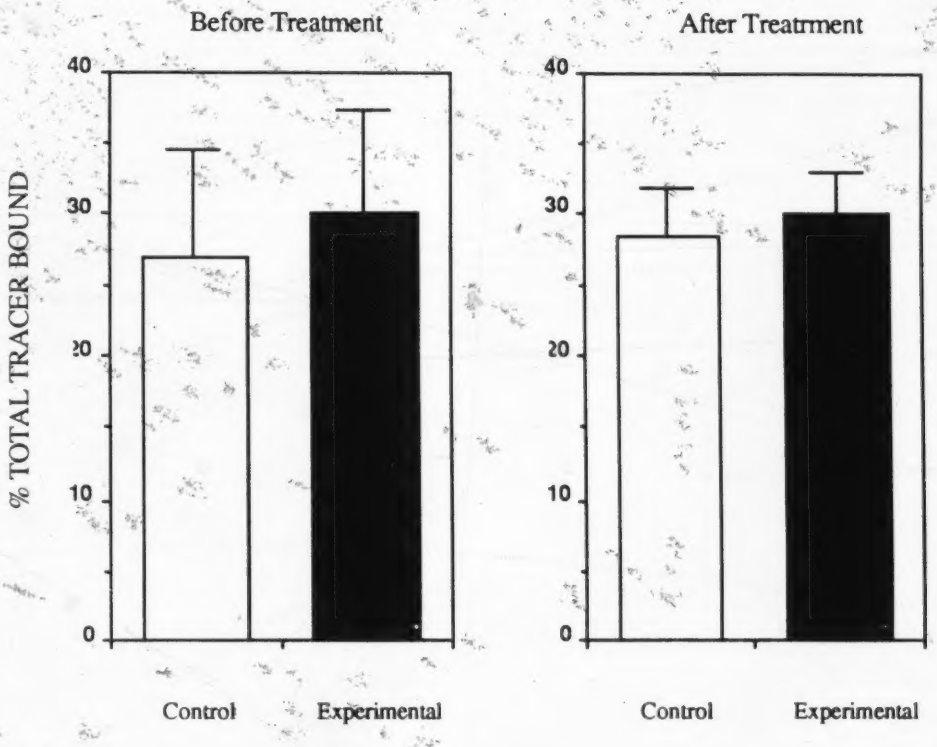

Before Treatment
After Treatrment
% TOTAL TRACER BOUND
40
30
20
10
0
Control
Experimental
Control
Experimental

Fig. 5.3 Effects of multiple injections of sGnRH-A on the serum GtH levels in sexually regressed goldfish (mixed sex). Fish received ip injections of PS (clear bars) or sGnRH-A (0.1 μg/g body weight, hatched bars) for the indicated time (at 7 day intervals). Blood samples were taken 6 hours following each injection. Significant differences between PS and sGnRH-A groups at each sample time are indicated by asterisks: *, $p<0.05$; **, $p< 0.01$ (Student's t test). Values are mean±SEM (n = 10).

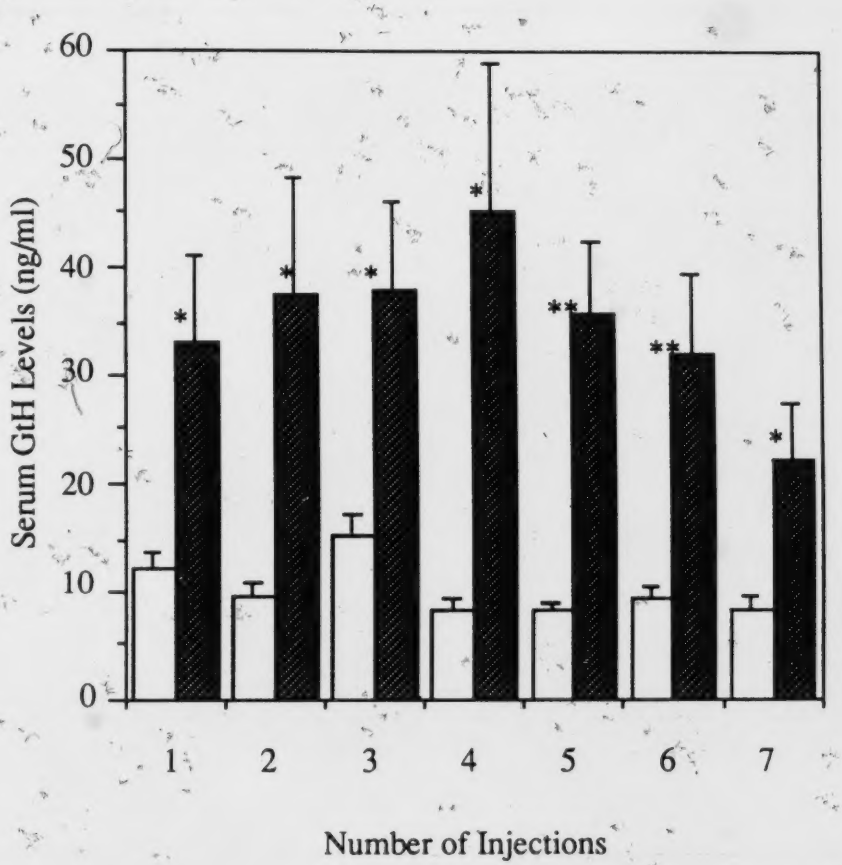

Serum GtH Levels (ng/ml)
60
50
40
30
20
10
0
1
2
3
4
5
6
7
Number of Injections

Fig. 5.4 Serum protein content of male goldfish at different stages of the reproductive cycle (sexually regressed, recrudescing, mature). Groups bearing the same letter are not significantly different ($p>0.05$, Duncan's Multiple Range test). Number in circles indicate the number of fish in each group. Values are mean±SEM.

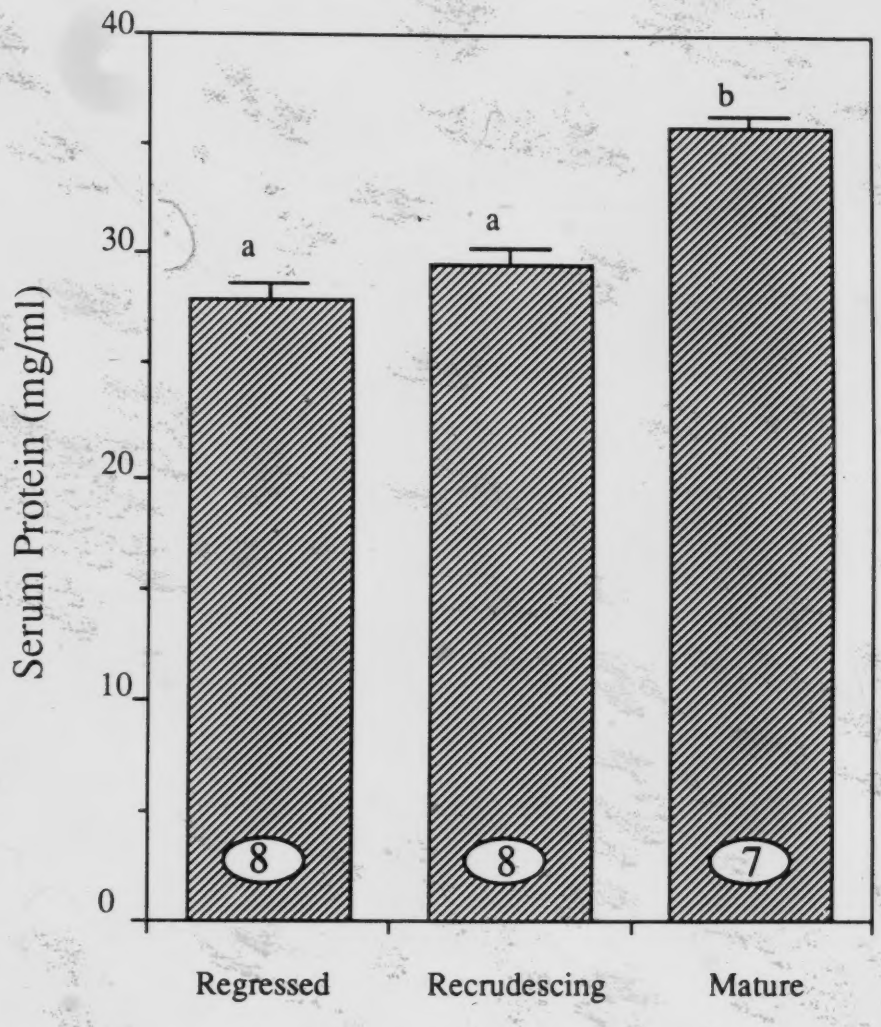

40
30
20
10
0
Serum Protein (mg/ml)
a
a
b
8
8
7
Regressed
Recrudescing
Mature

Fig. 5.5 Comparison of serum titer of GnRH-BP in male goldfish at different stages of the reproductive cycle (sexually regressed, recrudescing, mature). Individual samples were diluted 1 to 120 and tested in duplicate in the ligand binding assay. Number of fish in each group is indicated inside the circle. There were no significant differences between the groups ($p>0.05$, Mann-Whitney U test). Values are mean ± SEM.

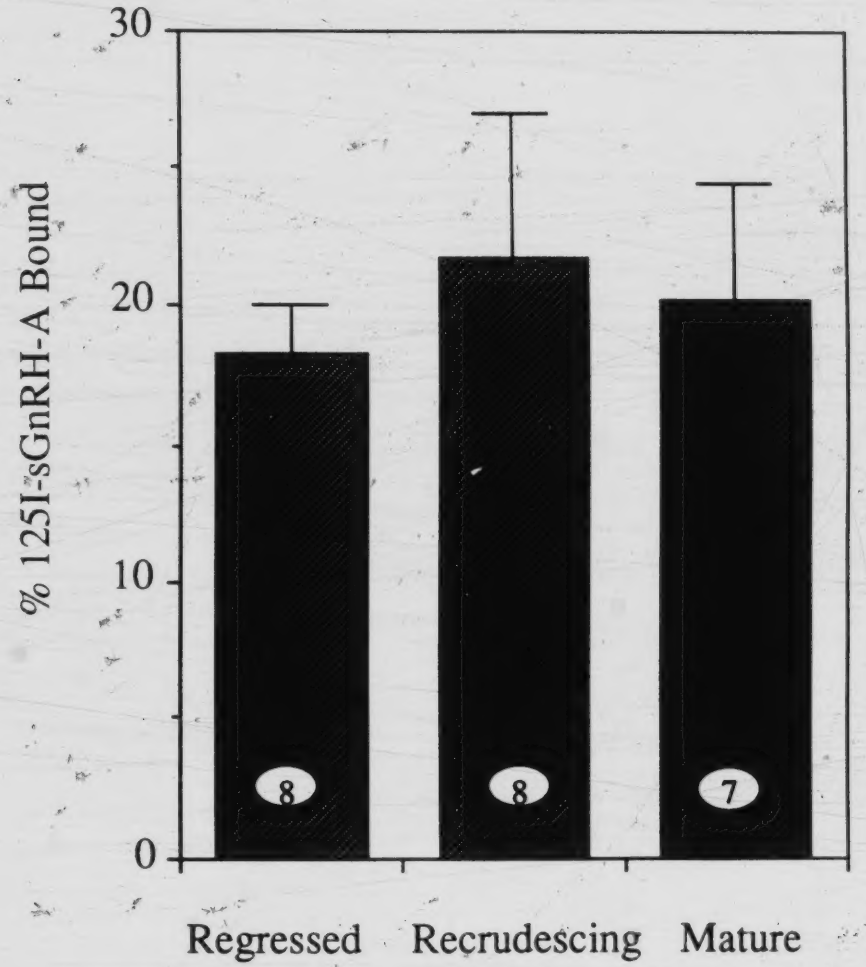

30
20
10
0
% 125I-sGnRH-A Bound
8
8
7
Regressed
Recrudescing
Mature

Fig. 5.6 Comparison of serum protein content of female goldfish at different stages of the reproductive cycle (sexually regressed, recrudescing, mature). Groups bearing the same letter are not significantly different ($p>0.05$, Duncan's test). Numbers in circles indicate the number of fish in each group. Values are mean±SEM.

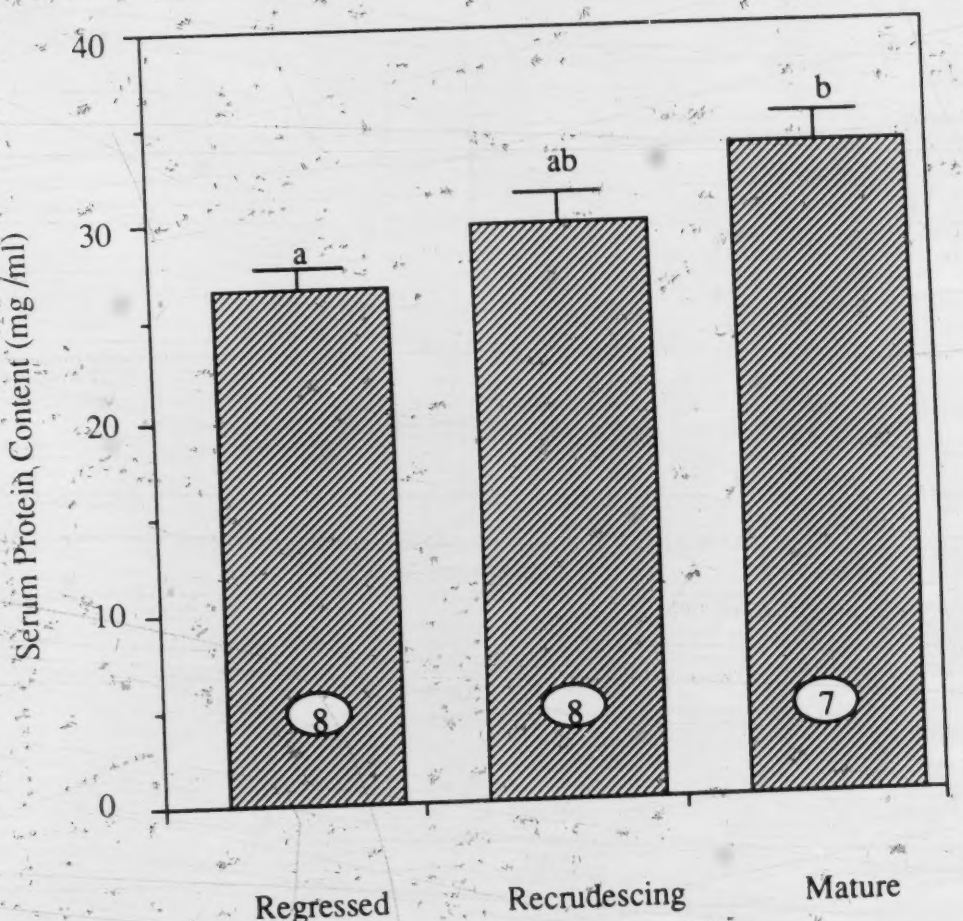

40
30
20
10
0
Serum Protein Content (mg /ml)
a
ab
b
8
8
7
Regressed
Recrudescing
Mature

Fig. 5.7 Comparison of serum titer of GnRH-BP in female goldfish at different stages of the reproductive cycle. Individual samples were diluted 1 : 120 and tested in duplicate in the ligand binding assay. Number of animals in each group is indicated in the circle. There were no significant differences between the groups ($p>0.05$, Mann-Whitney U test). Values are mean±SEM.

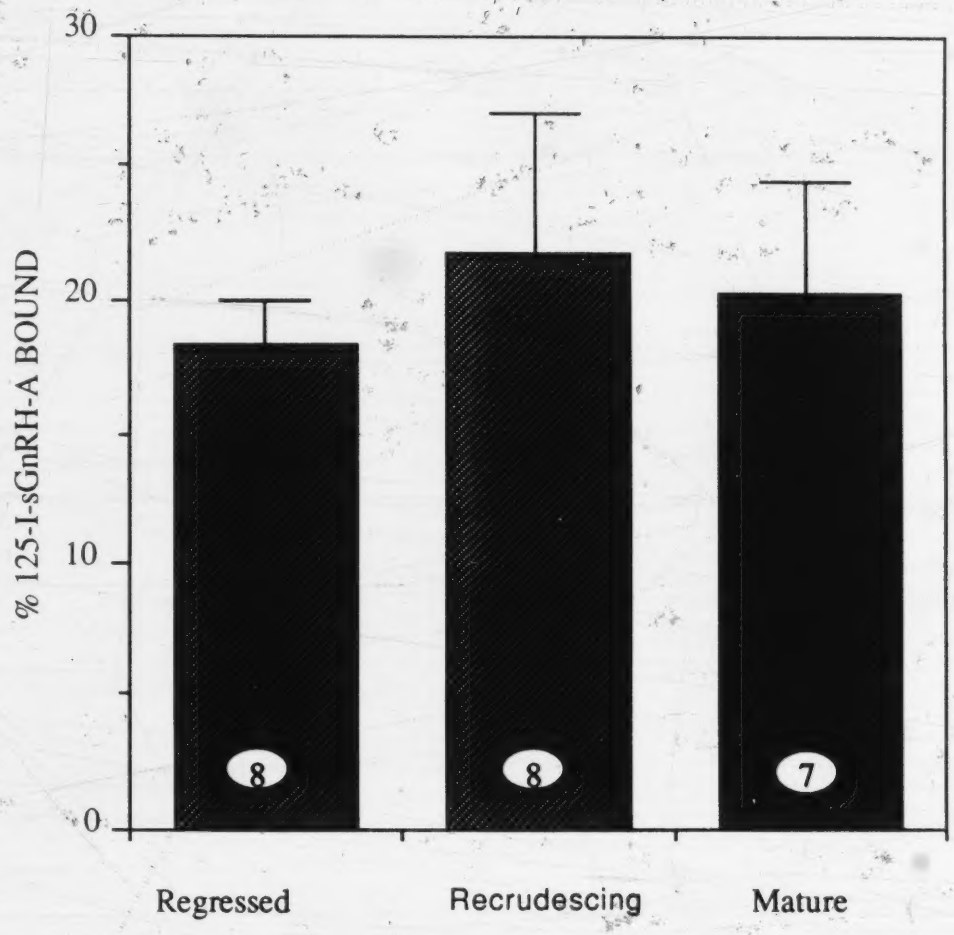

30
20
10
0
% 125-I-sGnRH-A BOUND
8
8
7
Regressed
Recrudescing
Mature

Fig. 5.8 Effect of E_2 implantation for 7 and 35 days on the serum protein content of sexually regressed female goldfish. Significant differences between the blank and E_2 implanted fish at each sample time are indicated by asterisks (***, $p<0.001$, Student's t test). Values are the mean±SEM (n =7).

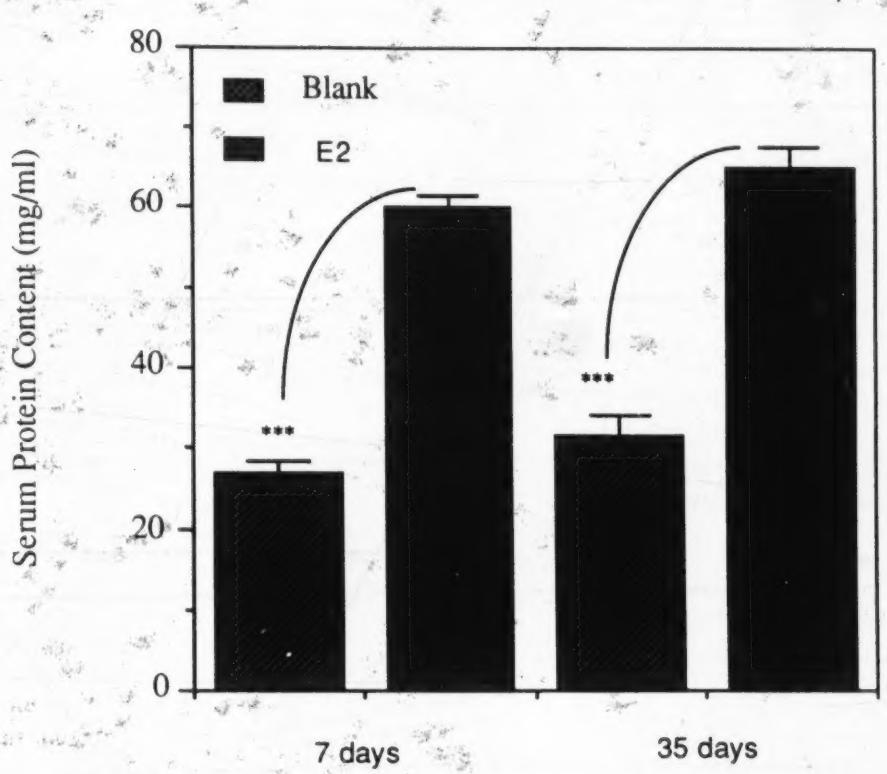
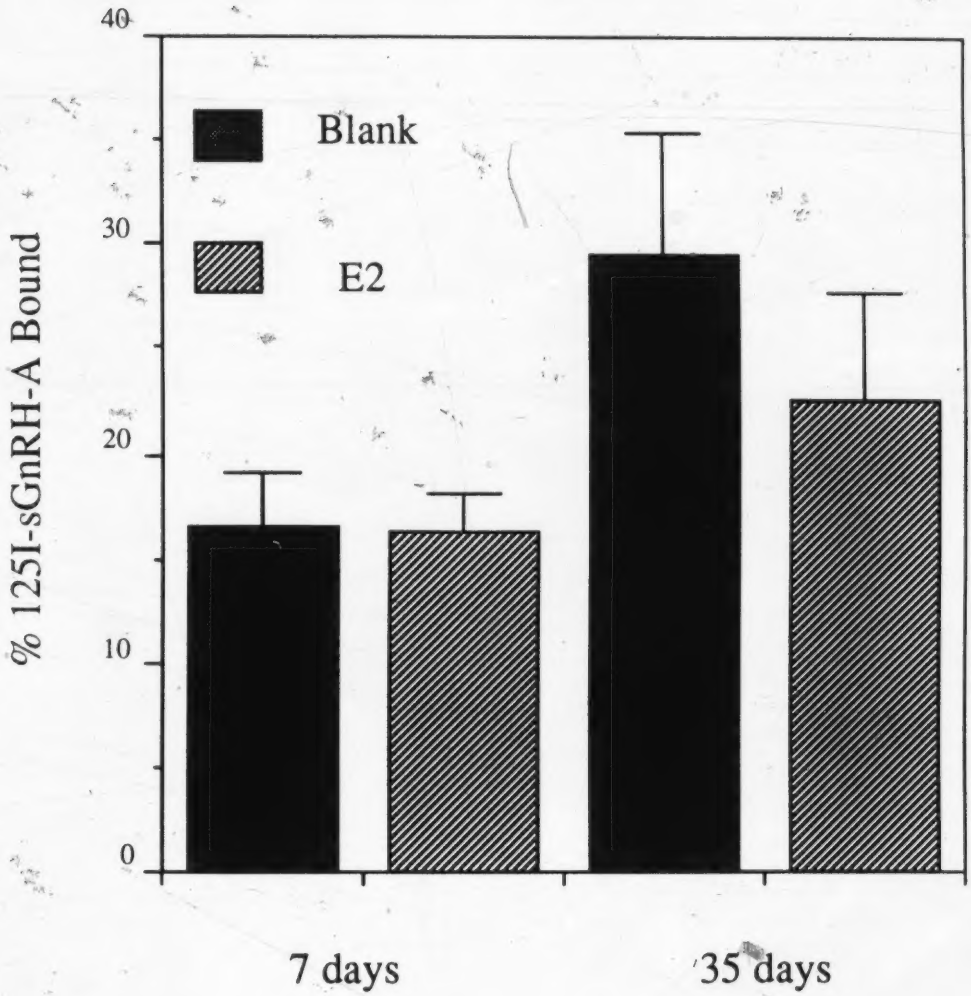

80
60
40
20
0
Blank
E2
Serum Protein Content (mg/ml)
***
***
7 days
35 days

Fig. 5.9 Effect of E_2 implantation on the serum titer of GnRH-BP. Serum samples were diluted 1 : 120 and tested in duplicate in the ligand binding assay. Therefore, the comparison of specific binding is based on a constant volume of diluted serum, not on per mg protein. There were no significant differences between the blank and E_2 implanted groups at either sample time (Mann-Whitney U test, $p>0.05$). Values are mean±SEM ($n = 7$).


40
30
20
10
0
% 125I-sGnRH-A Bound
Blank
E2
7 days
35 days

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6 METABOLISM OF GONADOTROPIN RELEASING HORMONES IN GOLDFISH: SERUM CLEARANCE AND TISSUE UPTAKE STUDIES.

6.1 INTRODUCTION

Gonadotropin (GtH) secretion in teleosts is under the dual regulation of gonadotropin-releasing hormone (GnRH) and dopamine, which inhibits GnRH-stimulated release (Peter *et al.*, 1986). On the basis of chromatographic and immunological studies, Yu *et al.* (1988) demonstrated the presence of [Trp^7, Leu^8]-GnRH (salmon GnRH, sGnRH) and [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II, cGnRH-II) in the brain and pituitary of the goldfish; a differential distribution of the two peptides was found, with about equal amounts in forebrain regions, and higher concentrations of cGnRH-II in mid- and hindbrain regions. The activity of several analogs of sGnRH and mammalian GnRH (mGnRH) have been studied in goldfish; [D-Arg^6, Pro^9-NEt]-sGnRH (sGnRH-A) was shown to be the most active analog both in vivo (Peter *et al.*, 1985) and in vitro (Habibi *et al.*, 1989b). This superactive analog has been successfully used in inducing spawning in Chinese carps (Lin *et al.*, 1988; Peter *et al.*, 1988), sea bass (Almendras *et al.*, 1988) and Chinese loach (Lin *et al.*, 1988).

Sherwood and Harvey (1986) used radioimmunoassay (RIA) to measure plasma GnRH levels following a single intraperitoneal (ip) injection of synthetic mammalian GnRH (mGnRH) in goldfish. Their results indicated that the rate of clearance from the plasma was rapid, as half of the hormone present at the peak (4 min after injection) had disappeared 12 min later. This initial half disappearance time ($T_{(1/2)i}$) of mGnRH in goldfish is slightly longer than that of mGnRH in mammalian species. In the rat the $T_{(1/2)i}$ after intravenous (iv) injection ranges from three to ten min (Miyachi *et al.*, 1973; Redding

and Schally, 1973; Swift and Crighton, 1979). There is no information available concerning the serum metabolic clearance rate (MCR) and initial distribution volume (V_i) of intra-arterially (ia) injected GnRH in teleost fishes. In the present study, the MCR, $T_{(1/2)i}$ and V_i of radioiodinated sGnRH and the superactive analog sGnRH-A were determined following a single ia injection. In addition, the tissue uptake and distribution of iodinated sGnRH-A was investigated to determine the sites of labeled hormone accumulation.

6.2 MATERIALS AND METHODS

Source and Maintenance of Fish

Goldfish, *Carassius auratus*, of the common or comet varieties were purchased from Grassyforks Fisheries Ltd., Martinsville, Indiana. All fish were maintained for a minimum of 28 days in 96-liter flow-through aquaria under a daily cycle of 16 hours light and 8 hours dark at 20±1 °C, prior to experimental treatment. The majority of the fish used (78%) were of 25-45g in body weight. At random times during the photophase the fish were fed a commercial fish chow (Ewos) to excess.

Preparation of Radioiodinated Gonadotropin-Releasing Hormones

Hormone clearance studies were performed with synthetic sGnRH and sGnRH-A, synthesized and kindly provided by J. E. Rivier and W. W. Vale (The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, San Diego, CA). The iodination procedure and the specific activity determination of ^{125}I-sGnRH were described in Chapter 3; sGnRH-A was iodinated using a modified chloramine T method as described previously (Habibi *et al.*, 1987). The specific activities of ^{125}I-sGnRH and ^{125}I-sGnRH-A were 780-1100 μCi/μg and 1100-1300 μCi/μg, respectively. The tracers were diluted with saline such that each 40 μl volume contained about 200,000 cpm and an estimated 100 pg GnRH. Intra-arterial (ia) injection of iodinated GnRHs was made with a 1-ml syringe fitted with a disposable 30-gauge x 1/2 in needle. Delivery of 20 μl volume was controlled by a BP 600-1 Dispenser device (Hamilton Co., Reno, NV).

Intra-arterial Injection Procedure

Fish were anesthetized in 0.05% tricaine methanesulfonate (TMS), and positioned with ventral side up in a rubber support. A midventral incision made through the hypaxial musculature from the junction of the opercular membranes to the base of the right pectoral fin exposed the pectoral girdle. Fine-point scissors were used to cut the pectoral girdle, exposing the pericardial cavity, and retractors were used to spread the pectoral girdle. The pericardium was teased with fine forceps to expose the bulbous arteriosus. The injection was made into the bulbous arteriosus, selected as the injection site because of its thick musculature and volume compensatory function. After injecting the volume of labeled GnRH, the needle was left in position for about 20 heart beats. The incision was filled with saline and closed with a single suture of 4.0 silk thread. In all cases, respiratory and locomotory movements were regained within one to three minutes of injection. Throughout the operative procedure, care was taken to prevent any excessive hemorrhage; fish showing arterial hemorrhage were discarded from the experiment. The entire operative procedure was routinely accomplished in less than 10 min. Blood samples were taken from individual fish at 5, 10, 15, 30, 45, 60 min after ia injection of ^{125}I-sGnRH, or 5, 10, 15, 30, 45, 60, 90, 120, 150 min after ia injection of ^{125}I-sGnRH-A. Duplicate 100 µl aliquots of serum were assayed for total radioactivity in a gamma scintillation counter (model MS 4/600, Micromedic Systems Inc. Ltd, Horsham, PA) with a counting efficiency of about 75% for ^{125}I. The total radioactivity determined in the serum was expressed as a percentage of the injected total radioactivity per milliliter serum after standardization to 35g body weight. The latter calculation assumes the kinetics of ^{125}I-sGnRH and ^{125}I-sGnRH-A in goldfish to be linearly related to body weight.

Tissue Uptake of ^{125}I-sGnRH-A

At time intervals (30 and 120 min) after ia injection of 0.3 μCi of ^{125}I-sGnRH-A alone or in combination with 2 μg unlabeled sGnRH-A, the fish was anesthetized with TMS, a blood sample taken, and killed by cutting the spinal cord behind the head. Different organs were removed, rinsed in saline, blotted on absorbent paper, and weighed individually in pre-weighed test tubes. The radioactivity of each sample was determined in the gamma scintillation counter, and the results expressed as percentage of the total injected dose of radioactivity per gram tissue or per ml serum after standardized to a 35 g body weight.

Data Analysis

The disappearance curve, or metabolic clearance profile (MCP), expressed as a percentage of administered dose per milliliter serum, was plotted against time (x) on semilogarithmic paper. The initial part of the MCPs between 5 and 150 min for ^{125}I-sGnRH-A, or between 5 and 60 min for ^{125}I-sGnRH was linear in semilogarithmic plot. A regression line was then fitted by a computer program (StatWorks™ Cricket Software Version 1.1, 1985). The MCP of labeled GnRH in the goldfish serum was expressed by the equation:

$$Y(x) = A\ e^{-\alpha x}$$

where the concentration of tracer at any time after ia injection, Y(x), is a function of the ordinate intercept A and the slope α. The values of these parameters were determined using regression analysis of the computer program. The serum $T_{(1/2)i}$, MCR and Vi of injected hormone were calculated as 0.693/α, α/A and 1/A, respectively (Shipley and Clark, 1972; Tait *et al.*, 1961). Statistical differences between groups were determined

by analysis of variance and the Duncan's multiple range test, or the Mann-Whitney U-test where applicable (Steel and Torrie, 1960).

6.3 RESULTS

Fig. 6.1 illustrates the rate of disappearance of ^{125}I-sGnRH-A injected ia from the serum of a sexually mature female goldfish. When the logarithm of radioactivity was ploted against time, the disappearance curve was linear from 5 to 150 min post-injection, during which time over 70% of the radioactivity disappeared from the circulation. This serum disappearance profile of ia injected ^{125}I-sGnRH-A showed the typical characteristics of an exponential curve, which could be described by the following computer fitted line:

$Y(x) = 49,576\ e^{-0.0095x}$.

The time course of the serum levels of injected ^{125}I-sGnRH in sexually mature female goldfish is represented in Fig. 6.2. The disappearance curve was linear up to 60 min post-injection , and could be described as

$Y(x) = 18.2505\ e^{-0.0214x}$.

The injected ^{125}I-sGnRH disappeared from the circulation more rapidly compared with injected ^{125}I-sGnRH-A (Fig. 6.1 and Fig. 6.2). A similar experiment using ^{125}I-sGnRH with extended sampling times revealed a much slower decaying second component of the curve, which could be described by a two-compartment model (data not shown). The calculated values of MCR (Table 6.1) for ^{125}I-sGnRH-A and ^{125}I-sGnRH were obtained without making assumptions about the number and the nature of the compartments exchanging with the circulating ^{125}I-GnRHs. The values of initial half disappearance time $T_{(1/2)i}$ and initial distribution volume V_i are included (Table 6.1) to facilitate comparison with other clearance studies.

Results of tissue uptake of ^{125}I-sGnRH-A in female goldfish at 30 min post-injection are shown in Fig. 6.3. Serum contained the highest concentrations of ^{125}I-sGnRH-A for tracer injected alone or tracer injected with cold hormone. High levels of accumulated tracer were found in the gill, kidney and liver compared to air bladder, brain, gonad, eyes

and muscle (Fig. 6.3). Co-injection of excess cold sGnRH-A with ^{125}I-sGnRH-A caused a significant increase in the accumulated tracer in the gills, heart and liver, and a tendency for increased accumulation in the kidney. Co-injection of excess cold sGnRH-A caused a significant decrease in uptake of ^{125}I-sGnRH-A in the pituitary. At 120 min post-injection there was significantly less tracer accumulated in serum and heart compared to at 30 min. At 120 min co-injection of excess cold sGnRH-A caused a significant increase in accumulation of tracer in air bladder, kidney and liver; whereas, in the pituitary the excess cold sGnRH-A caused a significant displacement of the tracer (Fig. 6.4). Fig. 6.5 shows the results of tissue uptake of ^{125}I-sGnRH-A in mature male goldfish at 120 min post-injection. Serum contained the highest concentration of ^{125}I-sGnRH-A for tracer injected in combination with cold hormone, while pituitary accumulated the highest concentration of ^{125}I-sGnRH-A for tracer injected alone. Co-injection of excess cold sGnRH-A caused a significant increase in the accumulated tracer in the air bladder, brain, gill, heart, kidney, muscle and serum, whereas co-injection of excess cold sGnRH-A caused a significant decrease in uptake of ^{125}I-sGnRH-A in the pituitary.

6.4 DISCUSSION

The distribution of ia-injected iodinated sGnRH and sGnRH-A into various body compartments, enzymatic inactivation by tissues and excretion in the urine likely account for their gradual disappearance from the blood of goldfish. Calculation of the disappearance rates of sGnRH and sGnRH-A from serum, revealed a MCR of 0.1173 ml min^{-1} $35g^{-1}$ and a $T_{(1/2)i}$ of 32.38 min for sGnRH, and a MCR of 0.0192 ml min^{-1} $35g^{-1}$ and a $T_{(1/2)i}$ of 72.95 min for sGnRH-A. The $T_{(1/2)i}$ values of both sGnRH and sGnRH-A are longer than that of mGnRH in goldfish (less than 12 min; Sherwood and Harvey, 1986) or rat, where the $T_{(1/2)i}$ after iv injection ranges from three to ten min (Miyachi *et al.*, 1973; Redding and Schally, 1973; Swift and Crighton, 1979).

The presence of a specific GnRH binding protein in goldfish serum (Huang and Peter,1988) may play an important role in prolonging the $T_{(1/2)i}$ of both sGnRH and sGnRH-A in circulation. The GnRH binding protein in goldfish serum has a higher affinity towards sGnRH-A than sGnRH (Huang and Peter, 1988), but it does not recognize mGnRH (Chapter 2), which may explain the much shorter $T_{(1/2)i}$ (less than 12 min) of mGnRH in goldfish plasma (Sherwood and Harvey, 1986). In general, there was good correlation between the affinity of the serum binding protein towards these three forms of GnRH and their half life in circulation in goldfish. It is of interest to note that sGnRH-A, which had a longer $T_{(1/2)i}$ than sGnRH and mGnRH, has a higher biological potency and a longer duration of action than sGnRH and mGnRH in stimulating GtH release in vivo (Peter *et al.*, 1985). When goldfish are pretreated with pimozide, a dopamine antagonist, the response of serum GtH levels to sGnRH-A injection is of greatest magnitude at 24 and 48 hr, varying from 6 to 18 times greater than the response to mGnRH and sGnRH. Without pretreatment with pimozide, the response to sGnRH-A is also of greater magnitude and longer duration compared with sGnRH or mGnRH (Peter *et al.*,

1985). This higher potency and longer action may be partially due to the longer half life of sGnRH-A in circulation.

Figure 6.3 to Figure 6.5 show that gill, heart, kidney, liver and serum generally accumulate a greater concentration of ^{125}I-sGnRH-A compared to air bladder, brain, gonad, eyes and muscle. The levels of radioactivity in serum and heart at 120 min were lower than at 30 min, suggesting the gradual removal of GnRH from the circulation. Accumulation of high levels of radioactivity, in the liver and kidney after the injection of iodinated sGnRH-A (Figure 6.3 to Figure 6.5), suggests that these organs were the major sites for the removal of GnRH from circulation. This argument is supported by the previous finding that rat kidney and liver as well as anterior pituitary preferentially accumulated a labeled mGnRH analog 1 hour after iv injection (Sandow *et al.*, 1977). More recently, Zohar et al. (1989) showed that kidney and liver as well as pituitary are important sites for GnRH degradation in sea bream. The role of the gills in the clearance of circulating GnRH is not known; however, the high vascularization of the gill could partially explain the high concentration of radioactivity accumulated in this organ. In all three experiments, the pituitary was the only organ showing significant specific uptake of injected ^{125}I-sGnRH-A, confirming that the pituitary is the main target organ for GnRH action. This is also in good agreement with previous studies showing that the goldfish pituitary has specific GnRH receptors with limited capacity, and that the high affinity binding sites show increased capacity in mature goldfish (Habibi *et al.*, 1987, 1989a, b). Although there is evidence showing that GnRH has direct effects on gonadal functions in rat (Gore-Langton *et al.*, 1981; Hsueh and Erickson,1979; Hsueh and Ling, 1979), human (Tureck *et al.*, 1982) and goldfish (Habibi *et al.*,1988), we could not demonstrate specific uptake of ^{125}I-GnRH-A by gonads of mature male or female goldfish, perhaps due to the low density of specific binding sites relative to the large tissue mass of mature gonads.

Co-injection of excess cold sGnRH-A had a general tendency to increase the accumulated tracer in several tissues, such as serum, kidney, liver, and air bladder. Although there was no consistent pattern among the three experiments, the general tendency for excess cold hormone to keep tracer in these tissues longer suggests that the capacity of the excretion process for GnRH was partially saturated by the excess amount of cold hormone.

Because of the direct innervation of the teleost pituitary by GnRH fibers, the importance of endogenous circulating GnRH in goldfish is unclear. However, successful application of exogenous GnRH and GnRH analogs in inducing ovulation and spawning in many teleost species including goldfish (Almendras *et al.*, 1988; Carolsfeld *et al.*, 1988; Crim *et al.*,1986; Lee *et al.*, 1987; Lin *et al.*, 1988; Peter *et al.*, 1985, 1988), and the uptake into circulation of exogenous GnRH applied in different ways (Sherwood and Harvey,1986; and present study) indicate that GnRH in circulation certainly is effective in stimulating GtH release from the pituitary. Although this is not a particularly new conclusion, the fact that sGnRH and cGnRH-II are found in the serum of goldfish indicates that circulating GnRH may normally have an important physiological function (Peter *et al.*, 1990).

TABLE 6.1

Comparison of Initial Distribution Volume, Metabolic Clearance Rate, and Initial Half-Disappearance Time of 125I-sGnRH-A and 125I-sGnRH in Mature Female Goldfish (GSI =7.01 ± 0.65%), at T = 20 ± 1 °C

Tracer	Initial Ditribution Volume(ml)	Metabolic Clearance Rate (ml min^{-1} $35g^{-1}$)	Initial half-Disappearance Time (min)
125-I-sGnRG-A	2.017	0.0192	72.95
125-I-sGnRH	5.48	0.1173	32.38

Fig. 6.1 Serum disappearance of ^{125}I-sGnRH-A in sexually mature female goldfish maintained at 20±1 °C. Data were standardized to a body weight of 35g, and expressed as a percentage of the total tracer injected. Initial half-disappearance time ($T_{(1/2)i}$) =72.95 min; initial distribution volume (Vi)=2.017 ml; Metabolic Clearance Rate (MCR)=0.0192 ml min^{-1} $35g^{-1}$.

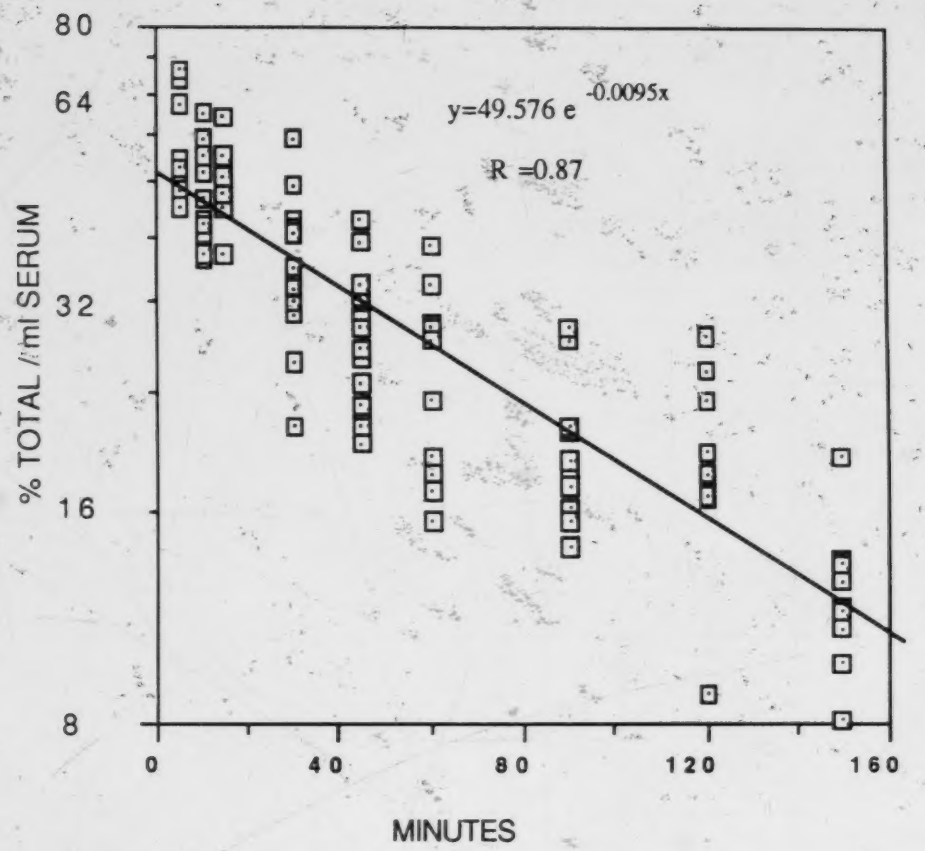

80
64
32
16
8
% TOTAL /ml SERUM
y=49.576 e -0.0095x
R =0.87
0
40
80
120
160
MINUTES

Fig. 6.2. Serum disappearance of ^{125}I-sGnRH in sexually mature female goldfish maitained at 20±1·°C. Data were standardized to a body weight of 35g , and expressed as a percentage of the total tracer injected. $T_{(1/2)i}$=32.38 min; Vi=5.48ml; MCR=0.1173 ml min^{-1} $35g^{-1}$.

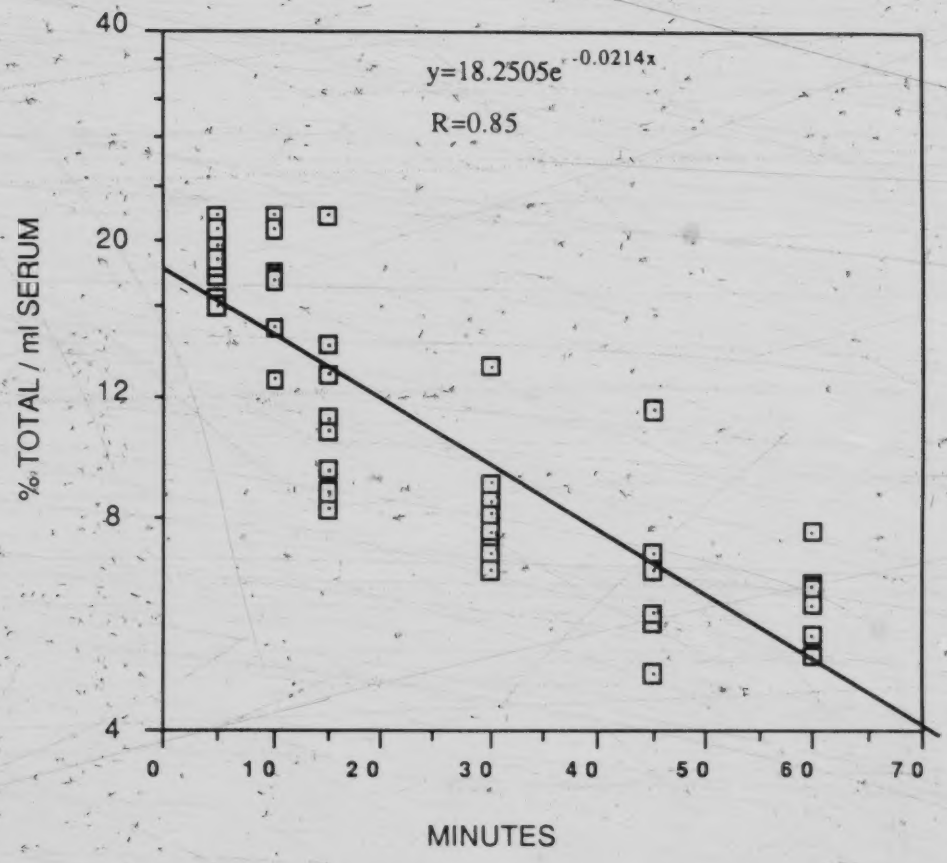

y=18.2505e -0.0214x
R=0.85
% TOTAL / ml SERUM
MINUTES
40
20
12
8
4
0
10
20
30
40
50
60
70

Fig. 6.3. Tissue uptake of ^{125}I-sGnRH-A at 30 min postinjection in sexually mature goldfish held at 20 °C. Each fish received a single intra-arterial injection of 0.5 μ Ci of ^{125}I-sGnRH-A (specific activity about 1200μ Ci / μg) alone or with 2 μg unlabeled sGnRH-A. Fish were killed with an overdose of tricaine methanesulfonate 30 minutes after the injection, and blood and other tissue samples taken immediately. Accumulated radioactivity was measured in a gamma spectrometer with a counting efficiency of 75% for ^{125}I. All data have been normalized to a standard body weight of 35 g. Data are mean±SEM (n= 8-10; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; Mann-Whitney U test). (A: air bladder; B: brain; D: gonad; E: eye; G: gill; H: heart; K: kidney; L: liver; M: muscle; P: pituitary; S: serum).

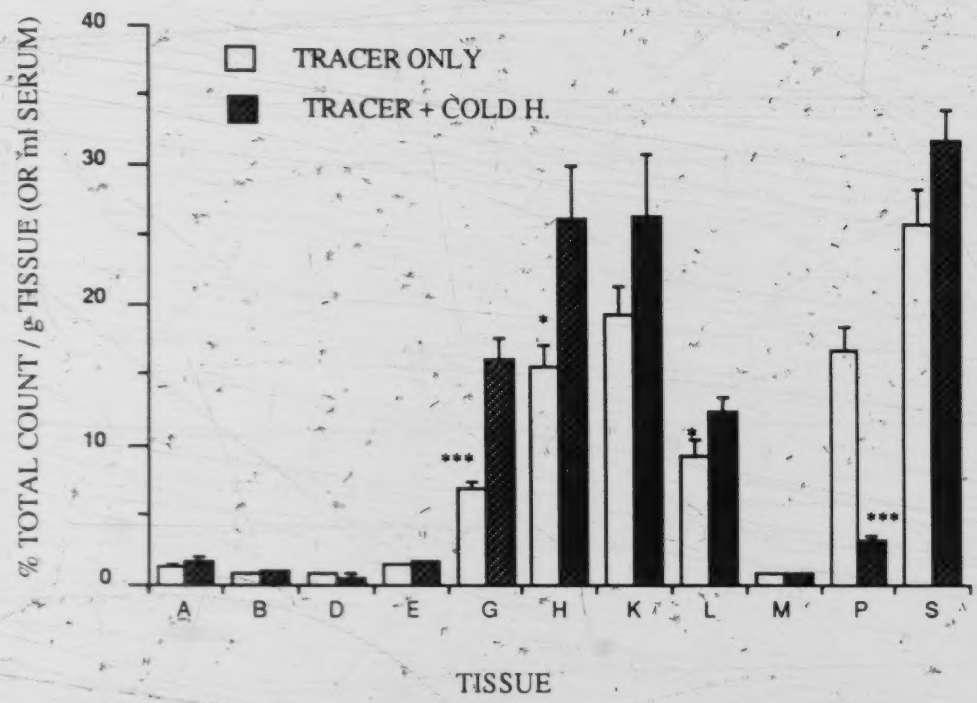
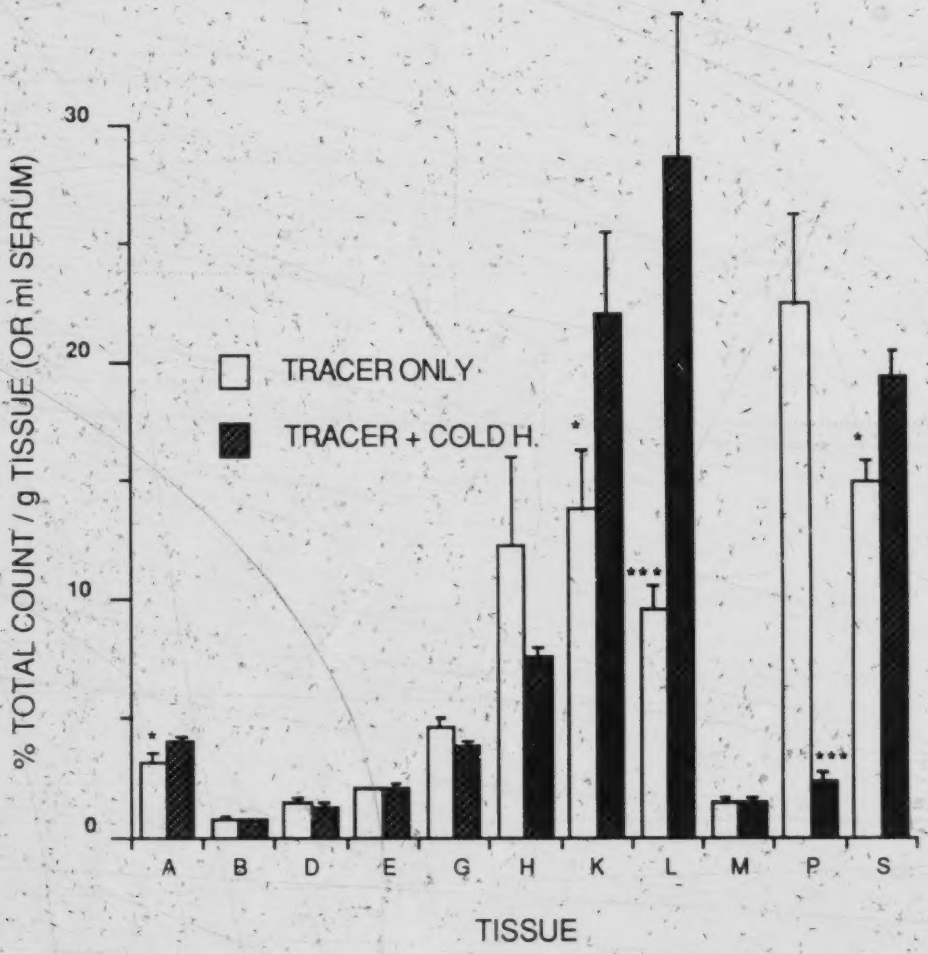

% TOTAL COUNT / g TISSUE (OR ml SERUM)
40
30
20
10
0
TRACER ONLY
TRACER + COLD H.
***
*
*
***
A
B
D
E
G
H
K
L
M
P
S
TISSUE

Fig. 6.4. Tissue uptake of ^{125}I-sGnRH-A at 120 min postinjection in sexually mature female goldfish. See caption to Fig. 6.3 for explanation of experiment, and symbols.


30
20
10
0
% TOTAL COUNT / g TISSUE (OR ml SERUM)
TRACER ONLY
TRACER + COLD H.
*
*
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A
B
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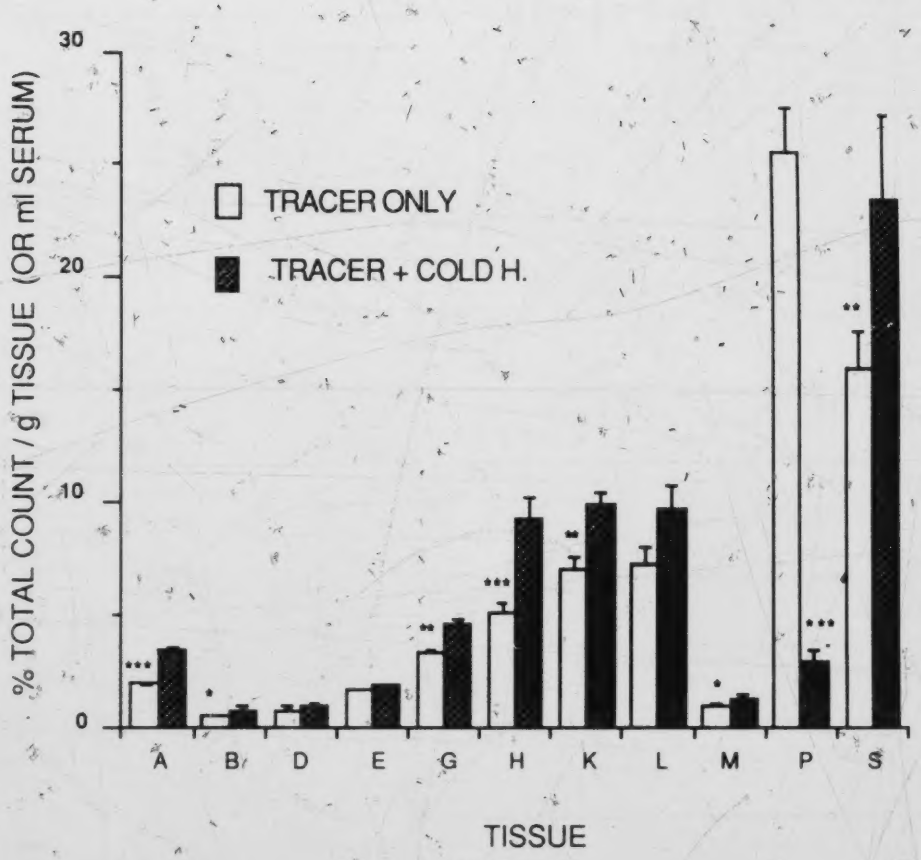
TISSUE

Fig. 6.5. Tissue uptake of ^{125}I-sGnRH-A at 120 min postinjection in sexually mature male goldfish. See caption to Fig. 6.3 for explanation of experiment, and symbols.


30
20
10
0
% TOTAL COUNT / g TISSUE (OR ml SERUM)
TRACER ONLY
TRACER + COLD H.
A
B
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TISSUE

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7. GENERAL DISCUSSION

Studies on the gonadotropin-releasing hormone (GnRH) binding protein (GnRH-BP) and metabolism of GnRH in goldfish are described in Chapter 2 through Chapter 6; this chapter presents a summary and general discussion of the findings.

The isolation and characterization of the GnRH-BP provide strong evidence for the presence of a specific carrier protein for GnRH in goldfish serum. In contrast to the reported nonspecific albumin binding of mammalian GnRH (mGnRH) and mGnRH analog (mGnRH-A) in human plasma (Tharandt *et al.*, 1979), the binding of sGnRH and [D-Arg^6, Pro^9-NEt]-sGnRH (sGnRH-A) in goldfish serum was due to a specific binding protein (Chapter 2). This specific binding in goldfish serum was dose dependent, time dependent, and reversible. The GnRH-BP was separated from the major components of serum proteins, including serum albumin, by a native polyacrylamide gel electrophoresis (Chapter 3). As described in Chapter 3, this native gel was a simple and effective way to facilitate the identification of the protein molecule responsible for the GnRH binding in goldfish serum. On the native gel, a single minor band of serum protein was isolated and identified as the only component responsible for the total GnRH binding in goldfish serum. Other protein bands, even at much higher concentrations, did not cause any increased binding compared to the nonspecific BSA binding (Chapter 3).

Unlike the GnRH receptors in goldfish pituitary, which recognizes mGnRH, chicken-I-GnRH (cGnRH-I), chicken-II-GnRH (cGnRH-II) as well as sGnRH (Habibi *et al.*, 1989b; H. Habibi, personal communication), the serum GnRH-BP recognized sGnRH and cGnRH-II, native forms of GnRH found in goldfish brain, pituitary, and serum (Yu *et al.*, 1988; Peter *et al.*, 1990; Chapter 4); but not mGnRH, cGnRH-I or lGnRH. Notably, the GnRH-BP also recognized analogs of sGnRH forms, but not mGnRH forms (Chapter 3). This different specificity between the serum binding protein and pituitary receptors argues

against the speculation that the circulating carrier protein might represent the GnRH receptor or a subunit thereof, although it is known that peptide hormone receptors can be shed from the cell membrane into the surrounding medium in vitro (Baumann and MacCart, 1984; McGuffin *et al.*, 1976). Other properties of the GnRH-BP also suggest that it is not a pituitary GnRH receptor. First, the affinities of the serum binding protein for sGnRH and sGnRH-A (Chapters 2 and 3; Huang and Peter, 1988) are lower than the affinities of the pituitary receptor for these two peptides (Habibi *et al.*, 1987, 1989b). Second, the molecular weight of the binding protein does not agree with the molecular weight estimates for GnRH binding sites in the goldfish pituitary (H. Habibi, R. Peter and E. Hazum, personal communication). Finally, in view of the relatively low capacity of the specific binding of GnRH in the goldfish pituitary (Habibi *et al.*, 1987), it seems unlikely that shed receptors could explain the capacity of the serum GnRH-BP. The serum GnRH-BP is more likely a molecule different from the pituitary receptor.

The finding of a GnRH-BP in goldfish serum is a recent addition to the growing number of circulating peptide hormone carrier proteins, including insulin-like growth factor (IGF) binding proteins (IGF-BP), human growth hormone binding protein (GH-BP) and corticotropin-releasing factor (CRF) binding protein (CRF-BP). Most of the published studies have approached the characterization of circulating peptide binding proteins in one or a combination of the following ways: evaluation of the elution pattern of radioactivity from a gel filtration column (Binoux *et al.*, 1982; Borsi *et al.*, 1982; Chatelain *et al.*, 1983; Clemmons *et al.*, 1983; Cohen and Blethen, 1983; Copeland *et al.*, 1980; Furlanetto, 1980; Hintz and Liu, 1977; Hintz *et al.*, 1981; Huang and Peter, 1988; Kaufmann *et al.*, 1977, 1978; Linton *et al.*, 1988; Morris and Schalch, 1982; Moses *et al.*, 1976, 1979; Orth and Mount, 1987; Schalch *et al.*, 1982; White *et al.*, 1981; Wilkins and D'Ercole, 1985; Zapf *et al.*, 1975), assessment of binding activity by activated charcoal separation of free and bound labeled peptide after its incubation with serum

(Binoux *et al.*, 1982; Borsi *et al.*, 1982; Clemmons *et al.*, 1983; Cohen and Blethen, 1983; Daughaday *et al.*, 1982; Hintz *et al.*, 1981; Huang and Peter, 1988; Kaufmann *et al.*, 1977, 1978; Moses *et al.*, 1976, 1979; Schalch *et al.*, 1982; White *et al.*, 1981; Wilkins and D'Ercole, 1985; Zapf *et al.*, 1975), or affinity-labeling of circulating binding proteins using the cross-linking reagent disuccinimidyl suberate (DSS) (D'Ercole and Wilkins, 1984; Grizzard *et al.*, 1984; Orth and Mount, 1988; Wilkins and D'Ercole, 1985). In this study, similar approaches were used. Sephadex G-50 mini-columns were used to separate the bound labeled ligand from free, which enabled the characterization of the dose-dependent binding, and the association and dissociation time courses, as well as the displacement curves. The dissociation of the ligand-protein complex is low in the mini-column separation system (Yoshimi *and Lipsett.*, 1968). Although the binding affinity of this GnRH-BP is low, the use of a pre-cooled mini-column, pre-cooled elution buffer, and very short elution time minimized the possible dissociation during the separation procedure (Chapter 2; Huang and Peter, 1988).

The goldfish serum GnRH-BP is of lower affinity compared with the high-affinity, low capacity binding sites in the goldfish pituitary, which have been suggested to be involved in the regulation of gonadotropin (GtH) release (Habibi *et al.*, 1987, 1989b). Although the GnRH-BP in goldfish serum is more abundant than the high affinity binding sites in the pituitary (Habibi et al., 1987, 1989b), the lower affinity of GnRH-BP and the relatively rapid dissociation of the hormone-binding protein complex in the circulation would favor the transfer of GnRH into the pituitary and eventually, full occupancy of pituitary receptor sites.

The use of affinity labeling enabled the visualization of the GnRH-BP when diluted goldfish serum or GnRH-BP partially purified by native gel was affinity-labeled with ^{125}I-sGnRH-L by DSS. The covalently linked radiolabeled complexes are stable under denaturing conditions, which enabled estimation of molecular size by SDS-polyacrylamide

gel electrophoresis (PAGE) and autoradiography. The estimated molecular weight of the GnRH-BP was approximately 40K dalton (Chapter 3); the estimated molecular weights for the pituitary GnRH receptors are 71K and 130K dalton (low affinity binding site) and 58K dalton (high affinity binding site), respectively (H. Habibi, R. Peter, E. Hazum, personal communication). Results in Chapters 3 and 5 exclude the major components of plasma proteins, such as albumin or vitellogenin, as being responsible for GnRH binding in goldfish serum.

In addition to the well established IGF binding proteins, some other newly characterized carrier proteins for peptide hormones have been reported in recent years. Like human CRF-BP, which recognizes human CRF-41, but not ovine CRF-41(Orth and Mount, 1987),the GnRH-BP in goldfish serum only recognized sGnRH and cGnRH-II, the native forms of GnRH in goldfish (Yu *et al.*, 1987, 1988), but not mGnRH, cGnRH-I, or lamprey GnRH (lGnRH), the other known forms of naturally occurring GnRH molecules. It is also interesting that a specific GnRH-BP is present in goldfish serum, but no specific GnRH-BP is found in human serum (Tharandt *et al.*, 1979). Similarly, CRF-BP is found in human, but not in sheep or rat (Linton *et al.*, 1988; Orth and Mount, 1987); GH-BP is found in human and rabbit blood, but not in rat blood (Bauman *et al.*, 1987; Leung *et al.*, 1987). This kind of species specificity may suggest a specific physiological function in the species where the binding protein is present.

As discussed in Chapter 4, the demonstration of cGnRH-II and sGnRH in goldfish serum is in good agreement with previous studies showing these two forms are present in goldfish brain, spinal cord and pituitary extracts (Yu *et al.*, 1987, 1988). Although antiserum S-30-3 used in the sGnRH RIA crossreacted substantially with mGnRH and cGnRH-I, these peptide were not found in the goldfish serum. The finding of two forms of GnRH in the circulation of a vertebrate is the first such case reported. In chicken both cGnRH-I and cGnRH-II are present in the brain, but only cGnRH-I is present in the median eminence (Mikami *et al.*, 1988; Sharp *et al.*, 1988), suggesting that only

cGnRH-I is released into the portal blood system. On this basis, cGnRH-I is suggested to have a neuroendocrine function and cGnRH-II to have a neurotransmitter or neuromodulator function in chicken. In goldfish, a functional hypothalamo-hypophysial blood portal system is lacking and the pituitary is directly innervated by immunoreactive GnRH fibers (Kah *et al.*, 1984, 1986). The presence of a substantial amount of both cGnRH-II and sGnRH in the pituitary of goldfish (Yu *et al.*, 1988) suggests a role for both GnRH forms in the regulation of pituitary functions. It has been demonstrated that sGnRH, mGnRH , and analogs of each stimulate growth hormone as well as gonadotropin release in vivo as well as in vitro from perifused fragments of the goldfish pituitary (Marchant and Peter, 1989; Marchant *et al.*, 1989). More recently, it has been shown that cGnRH-II is more potent than sGnRH in releasing GtH, but equipotent in releasing GH, from cultured dispersed cells of the goldfish pituitary (Chang *et al.*, 1990; Peter *et al.*, 1990). The ED_{50} dosage for sGnRH and cGnRH-II in releasing GtH and GH in the *in vitro* test systems used is in the nanomolar range, with the lower ends of the dose response curves in the mid-picomolar range. Notably, the concentrations of sGnRH and cGnRH-II detected in goldfish serum coincides with the minimal dosages found effective in stimulating GH and GtH release *in vitro*. Although it is not known whether circulating sGnRH and cGnRH-II have a neuroendocrine function in goldfish, the present study has provided a starting point for further evaluation of the possible physiological function of circulating GnRH, particularly in regard to its roles as a hormone.

The origin of circulating GnRH is not known. Notably the serum concentrations of cGnRH-II are higher than that of sGnRH, in contrast to the pituitary where the concentrations of cGnRH-II are lower than that of sGnRH in male goldfish and in females where similar concentrations of both forms are found (Yu *et al.*, 1988). This suggests that the two forms of GnRH may be differentially released into the circulation. The mismatch between pituitary content and serum concentrations of the two forms of GnRH would

suggest that other origins may also contribute to the circulating pools of cGnRH-II and sGnRH.

In Chapters 2-4, all experiments were carried out using pooled serum samples. In Chapter 5, individual serum samples were collected and used in the ligand binding assay for determination of the serum titer of GnRH-BP. A measurable amount of GnRH-BP was consistently detected in every individual serum sample from both male and female goldfish; there were no significant differences in the serum titer of GnRH-BP between males and females. Similar observations were reported in human CRF-BP. CRF-BP was first demonstrated in late gestational maternal plasma (Linton and Lowry, 1986), where it coexists with substantially elevated plasma levels of CRF-41 (Schulte and Healy, 1985; Campbell *et al.*, 1987); subsequent studies demonstrated that the binding protein is also present in normal male plasma, where the endogenous CRF content is low (Linton *et al.*, 1988; Orth and Mount, 1987).

The GnRH-BP titer was not affected by long term treatment with sGnRH-A. Tharandt *et al.* (1979) also found no significant changes in the titer of nonspecific mGnRH binding in human serum after 2 months of treatment with a potent mGnRH analog. In both cases the most important result was the absence of any increase of binding of diluted sera, which might have pointed towards formation of an antibody to the analog. In the present study, the serum titer of GnRH-BP was not affected by the secondary effect of high levels of GtH resulting from prolonged stimulation by sGnRH-A. This is in contrast to the sex steroid binding globulin in human, where androgens and estrogens both can change the concentration of the binding protein, which in turn influences the delivery of sex steroids to both target and metabolizing tissues (Belgorosky and Rivarola, 1982).

As indicated in Chapter 3, the GnRH-BP is not a major component of goldfish serum proteins. Results in Chapter 5 show that sexually mature (completed gonadal recrudescence) male and female fish had a higher serum protein content than sexually

regressed fish, reflecting changes in some major components of serum proteins. However, the serum titer of GnRH-BP was not different between mature and regressed fish. This is in agreement with the previous findings that the GnRH binding in goldfish serum is not due to a major serum protein component, and that the GnRH-BP accounts for only a small portion of the total protein content in goldfish serum (Chapter 3). Unlike the changes in the total protein content, the serum titer of GnRH-BP did not change in different stages of the reproductive cycle in both males and females, suggesting that the titer of GnRH-BP is not fine tuned to the changes related to gonadal development. It is unlikely that the GnRH-BP levels will change rapidly, since it is already present with high capacity in goldfish serum (Chapter 2), and is not saturated by the low endogenous levels of GnRH detected in the circulation (Chapter 4).

When sexually regressed female goldfish were implanted with estradiol (E_2), the serum total protein content was doubled, but the serum titer of GnRH-BP did not change (Chapter 5). Since the vitellogenin (Vg) content in the serum samples was not measured, it is not known what proportion of the E_2 induced protein content (over 30 mg/ml) was contributed by Vg. However, it is well documented that Vg in fish is normally phospholipoglycoproteins, and their synthesis is induced in the liver by estrogens in both male and female fish (Wallace and Selman, 1981; Wiegand, 1982; Ng and Idler, 1983). Moreover, Vg has been claimed to be the only serum protein that is induced by estrogen in Xenopus (Wallace, 1970). The majority of the increased protein content in serum in the E_2-treated fish in the present study is likely due to the increased secretion of Vg by liver, and the absence of Vg incorporation into previtellogenic oocytes may favor the accumulation of high levels in the serum. In the literature, the levels of Vg reported range from 1 to 100 mg/ml serum (Crim and Idler, 1978; Goedmakers and Verboom, 1974; Sumpter, 1985). If Vg is assumed to be the major component induced by E_2 implant, then Vg is not associated with GnRH binding in goldfish serum because the serum titer of GnRH-BP did not increase when a large amount of Vg was accumulated in the serum.

The metabolic clearance rate (MCR) studies in Chapter 6 revealed a MCR of 0.1173 ml min^{-1} $35g^{-1}$ and a $T_{(1/2)i}$ of 32.38 min for sGnRH, and a MCR of 0.0192 ml min^{-1} $35g^{-1}$ and a initial half life ($T_{(1/2)i}$) of 72.95 min for sGnRH-A in goldfish. The $T_{(1/2)i}$ values of both sGnRH and sGnRH-A are longer than that of mGnRH in goldfish (less than 12 min; Sherwood and Harvey, 1986) or rat, where the $T_{(1/2)i}$ after iv injection ranges from three to ten min (Miyachi *et al.*, 1973; Redding and Schally, 1973; Swift and Crighton, 1979).

The presence of a specific GnRH binding protein in goldfish serum (Huang and Peter;1988) may play an important role in prolonging the $T_{(1/2)i}$ of both sGnRH and sGnRH-A in circulation. The GnRH binding protein in goldfish serum has a higher affinity towards sGnRH-A than sGnRH (Chapter 2; Huang and Peter, 1988), but it does not recognize mGnRH (Chapter 3), which may explain the much shorter $T_{(1/2)i}$ (less than 12 min) of mGnRH in goldfish plasma (Sherwood and Harvey, 1986). In general, there was good correlation between the affinity of the serum binding protein towards these three forms of GnRH and their half lives in circulation in goldfish.

It is of interest to note that sGnRH-A , which had a longer $T_{(1/2)i}$ than sGnRH and mGnRH, has a higher biological potency and a longer duration of action than sGnRH and mGnRH in stimulating GtH release *in vivo* in goldfish (Peter *et al.*, 1985). When goldfish are pretreated with pimozide, a dopamine antagonist, the response of serum GtH levels to sGnRH-A injection is of greatest magnitude at 24 and 48 hr, varying from 6 to 18 times greater than the response to mGnRH and sGnRH. Without pretreatment with pimozide, the response to sGnRH-A is also of greater magnitude and longer duration compared with sGnRH or mGnRH (Peter *et al.*, 1985). This higher potency and longer action may be partially due to the longer half life of sGnRH-A in circulation. Accumulation of high levels of radioactivity in the liver and kidney after the injection of iodinated sGnRH-A suggests that these organs are the major sites for the removal of GnRH from circulation. This argument is supported by the previous findings that rat kidney and liver

as well as anterior pituitary preferentially accumulated a labeled mGnRH analog one hour after iv injection (Sandow *et al.*, 1977). More recently, Zohar *et al.* (1989) showed that kidney and liver as well as pituitary are important sites for GnRH degradation in sea bream. Results in Chapter 6 indicate that pituitary was the only organ showing significant specific uptake of injected ^{125}I-sGnRH-A (displaceable by unlabeled sGnRH-A), confirming that the pituitary is the main target organ for GnRH action. This is also in good agreement with previous studies showing that the goldfish pituitary has specific GnRH receptors with limited capacity, and that the high affinity binding sites show increased capacity in mature goldfish (Habibi *et al.*, 1987, 1989a, 1989b). Although there is evidence showing that GnRH has direct effects on gonadal functions in rat (Gore-Langton *et al.*, 1981; Hsueh and Erickson,1979; Hsueh and Ling, 1979), human (Tureck *et al.*, 1982) and goldfish (Habibi *et al.*,1988), there was no specific uptake of ^{125}I-GnRH-A by gonads of mature male or female goldfish, probably due to the low density of specific binding sites relative to the large tissue mass.

Because of the direct innervation of the teleost pituitary by GnRH fibers, the importance of endogenous circulating GnRH and its binding protein in goldfish is unclear. However, successful application of exogenous GnRH and GnRH analogs in inducing ovulation and spawning in many teleost species including goldfish (Almendras *et al.*, 1988; Carolsfeld *et al.*, 1988; Crim *et al.*,1986; Lee *et al.*, 1987; Lin *et al.*, 1988; Peter *et al.*, 1985, 1988), and the uptake into the circulation and pituitary of exogenous GnRH (Chapter 6; Sherwood and Harvey, 1986) indicate that GnRH in circulation certainly is effective in stimulating GtH release from the pituitary. Although this is not a particularly new conclusion, the fact that sGnRH and cGnRH-II are found in the serum of goldfish indicates that circulating GnRH may normally have an important physiological function (Peter *et al.*, 1990).

A number of questions concerning the possible physiological role of the GnRH-BP in

goldfish serum remain to be examined. The cyclostome and elasmobranch fishes either lack a hypothalamo-hypophysial blood portal system, or have no vascular cannection by the portal system to the part of the pituitary containing gonadotrophs, respectively (Gorbman *et al.*, 1983). In these primitive fishes GnRH likely reaches pituitary gonadotrophs by the general circulation. However, in the goldfish gonadotrophs are directly innervated by GnRH neurons from hypothalamus, and in this situation the importance of maintaining a reservoir of GnRH in circulation is not as obvious as in the more primitive fishes. Perhaps the GnRH-BP is vestigial from the condition in the more primitive fishes.

As discussed above, IGF-binding protein can act as an autocrine/paracrine inhibitor of IGF action in the human endometrial tissue, and that the IGF-binding protein complexes in circulation are probably taken away from rather than delivered to their target. In goldfish, the GnRH-BP may function to carry GnRH away from the site of action, thereby preventing prolonged action and desensitization of receptors. Clarification of the above points await purification of the bioactive GnRH-BP to homogeneity before detailed studies can be carried out with confidence.

In summary, data in this thesis indicate the existence of a specific GnRH binding protein in goldfish serum, and its molecular weight is approximately 40K dalton.This GnRH-BP is specific for sGnRH and cGnRH-II, the native forms of GnRH in goldfish, but not for other natural forms of vertebrate GnRH. The presence of this GnRH-BP may prolong the half life of GnRH in circulation. However, its titer is not affected by high levels of E_2, GnRH or GnRH induced GtH. This GnRH-BP is present with high capacity in both males and females, coexisting with low concentrations of circulating GnRH, and consequently, unlikely to be closely regulated.

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8. Appendix A preliminary Study on The Origin of The GnRH Binding Protein

8.1 Materials and Methods

The procedure described in Chapter 3 for photoperiod-temperature regime and procedures for fish maintenance, fish handling, blood sampling, analytical gel and autoradiography were followed.

Tissues from brain, pituitary, testes, ovaries and liver were homogenized in 40mM phosphate buffer, pH 7.4. Isolated GnRH-BP (fraction C from preparative PAGE) or tissue homogenates or culture media of pituitary and telencephalon slices were incubated with approximately 200,000 cpm of ^{125}I-sGnRH-L in a final volume of 90 µl of 40 mM phosphate buffer, pH 7.4, for 10 hours at 4 °C. At the end of incubation, 10 µl of 5mM DSS, prepared freshly in dimethylsulfoxide, was added to the incubate to give a final concentration of 0.5 mM DSS. After 5 min at room temperature, the cross-liking reaction was quenched by the addition of 10 µl of 1.0 M Tris-HCl, pH 7.4. Samples were run on SDS-gel as described in Chapter 3. Ligand binding assay was carried out with liver homogenate and diluted serum following the procedures described for GnRH-BP binding test previously (Chapters 2 and 3).

8.2 Results and Discussion

Fig. A-1 shows an autoradiogram of affinity labeling of liver homogenate (lane 1), culture medium of telencephalon slices (lanes 2 and 4), isolated GnRH-BP (lane 3) and culture medium of pituitary slices (lanes 5 and 6) to ^{125}I-sGnRH-L. Only the homogenate of liver showed a positively labeled band similar to the GnRH-BP in molecular weight. Culture media of pituitary and telencephalon slices showed no positively labeled band similar to the GnRH-BP. Autoradiography of homogenates of ovary, testes or pituitary did not show a labeled band similar to the GnRH-BP (data not shown).

Fig. A-2 shows that liver homogenate had specific binding of ^{125}I-sGnRH-A after 30, 60 and 600 min of preincubation. Fig. A-3 shows that after 60 min of preincubation, both liver homogenate and serum had higher binding than BSA (Duncan's multiple range test $P<0.05$); and 10^{-5} M of sGnRH-A displaced the specific binding to the binding sites in liver homogenate and serum. Our preliminary data suggest that liver is the most likely candidate as the original organ of the GnRH-BP. More experiments are planned to further investigate this possibility.

A-1. Autoradiogram of liver homogenate (lane A), culture medium of telencephalon slices (lanes B), isolated GnRH-BP (lane C) and culture medium of pituitary slices (lane D) labeled with ^{125}I-sGnRH-L by affinity cross-linking. Only the homogenate of liver had positive affinity labeling of a 40K band similar to the GnRH-BP. Large amount of BSA present in the culture media caused nonspecific labeling of a 67K band.

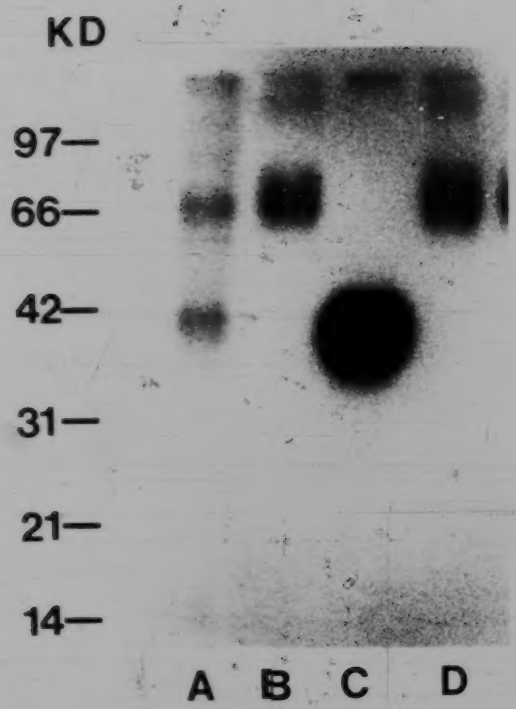

KD
97—
66—
42—
31—
21—
14—
A
B
C
D

Fig. A-2. Time course of ^{125}I-sGnRH-A binding with liver homogenate. Significant differences between BSA and liver homogenate sample at each time are indicated by asterisks: *, $p<0.05$; **, $p<0.01$ (Student's *t* test). Values are mean±SD (n=2).

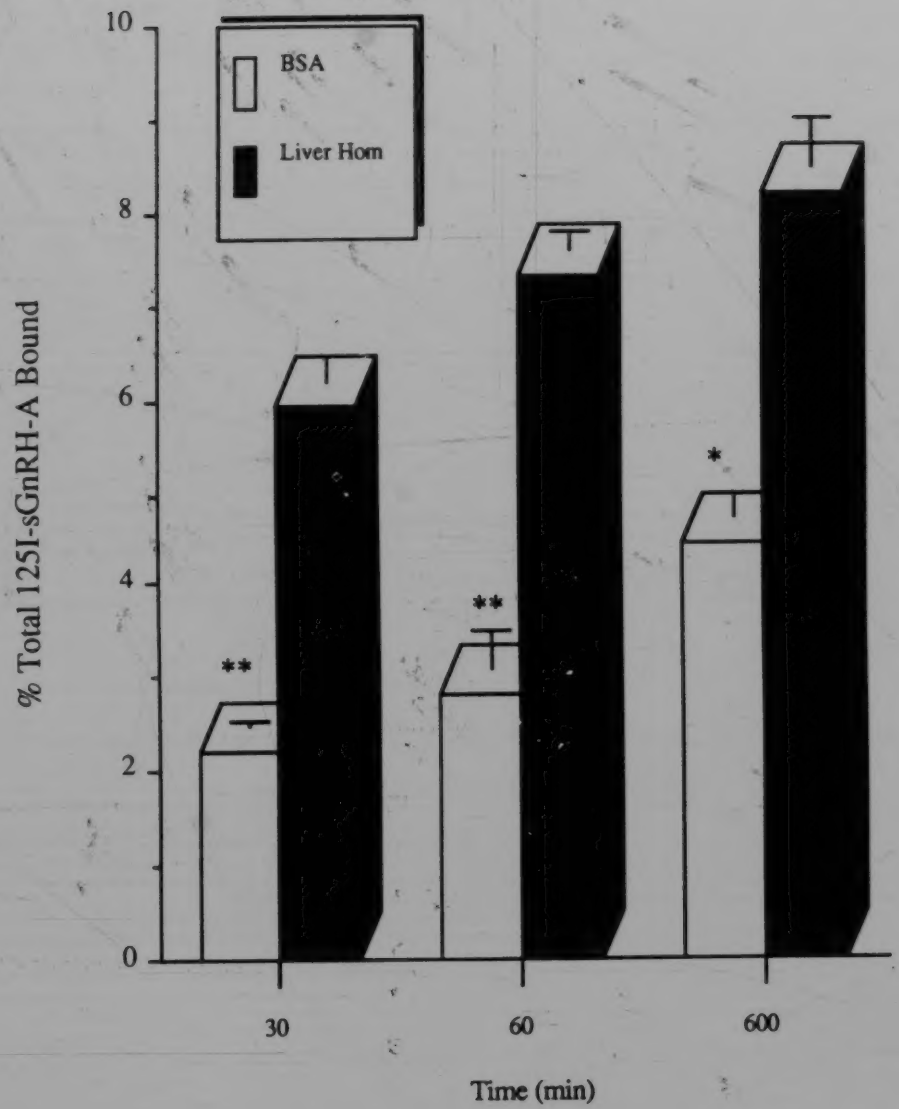
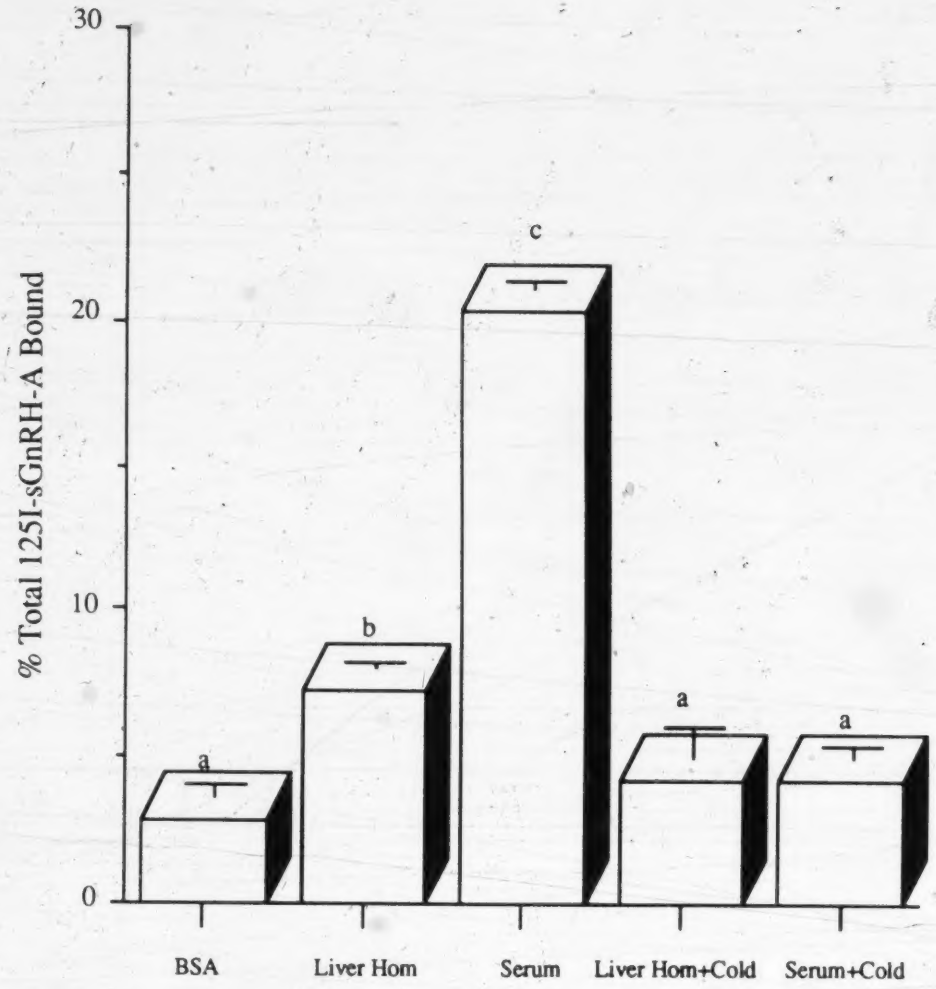

BSA
Liver Hom
% Total 125I-sGnRH-A Bound
10
8
6
4
2
0
**
**
*
30
60
600
Time (min)

Fig. A-3. Specific binding of ^{125}I-sGnRH-A to serum and liver homogenate.Unlabeled sGnRH-A , at 10^{-5} M, displaced tracer from serum and liver homogenate. Columns bearing the same letter are not significantly different ($p>0.05$, Duncan's multiple range test).Values are mean±SD (n=2).


30
20
10
0
% Total 125I-sGnRH-A Bound
a
b
c
a
a
BSA
Liver Hom
Serum
Liver Hom+Cold
Serum+Cold